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(21) International Application Number: PCT/US93/01956 (22) International Filing Date: 5 March 1993 (05.03.93) (30) Priority data: 07/846,349 5 March 1992 (05.03.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/846,349 (CIP) Filed on 5 March 1992 (05.03.92) (71) Applicants (for all designated States except US): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US); IMPERIAL CANCER RESEARCH TECHNOLOGY [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).		(72) Inventors; and (75) Inventors/Applicants (for US only) : THORPE, Philip, E. [GB/US]; 660 Preston Forest, #320, Dallas, TX 75230 (US). BURROWS, Francis, J. [GB/US]; 6346 Vickery, Dallas, TX 75214 (US). (74) Agent: PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i>
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DESCRIPTION

Diagnostic and/or therapeutic agents, targeted to neovascular endothelial cells.

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BACKGROUND OF THE INVENTION**1. Field of the Invention**

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The present invention relates generally to methods and compositions for targeting the vasculature of solid tumors using immunologically-based reagents. In particular aspects, antibodies carrying diagnostic or therapeutic agents are targeted to the vasculature of solid tumor masses through recognition of tumor vasculature-associated antigens, or through the specific induction of other antigens on vascular endothelial cells in solid tumors.

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2. Description of Related Art

Over the past 30 years, fundamental advances in the chemotherapy of neoplastic disease have been realized. While some progress has been made in the development of new chemotherapeutic agents, the more startling achievements have been made in the development of effective regimens for concurrent administration of drugs and our knowledge of the basic science, e.g., the underlying neoplastic processes at the cellular and tissue level, and the mechanism of action of basic antineoplastic agents. As a result of the fundamental achievement, we can point to significant advances in the chemotherapy of a number of neoplastic diseases, including choriocarcinoma, Wilm's tumor, acute leukemia, rhabdomyosarcoma, retinoblastoma, Hodgkin's disease and Burkitt's lymphoma, to name just a few. Despite the

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impressive advances that have been made in a few tumors, though, many of the most prevalent forms of human cancer still resist effective chemotherapeutic intervention.

5 The most significant underlying problem that must be addressed in any treatment regimen is the concept of "total cell kill." This concept holds that in order to have an effective treatment regimen, whether it be a surgical or chemotherapeutic approach or both, there must
10 be a total cell kill of all so-called "clonogenic" malignant cells, that is, cells that have the ability to grow uncontrolled and replace any tumor mass that might be removed. Due to the ultimate need to develop therapeutic agents and regimens that will achieve a total
15 cell kill, certain types of tumors have been more amenable than others to therapy. For example, the soft tissue tumors (e.g., lymphomas), and tumors of the blood and blood-forming organs (e.g., leukemias) have generally been more responsive to chemotherapeutic therapy than
20 have solid tumors such as carcinomas. One reason for this is the greater physical accessibility of lymphoma and leukemic cells to chemotherapeutic intervention. Simply put, it is much more difficult for most chemotherapeutic agents to reach all of the cells of a
25 solid tumor mass than it is the soft tumors and blood-based tumors, and therefore much more difficult to achieve a total cell kill. The toxicities associated with most conventional antitumor agents then become a limiting factor.

30

 A key to the development of successful antitumor agents is the ability to design agents that will selectively kill tumor cells, while exerting relatively little, if any, untoward effects against normal tissues.
35 This goal has been elusive to achieve, though, in that there are few qualitative differences between neoplastic and normal tissues. Because of this, much research over

the years has focused on identifying tumor-specific "marker antigens" that can serve as immunological targets both for chemotherapy and diagnosis. Many tumor-specific, or quasi-tumor-specific ("tumor-associated"), markers have been identified as tumor cell antigens that can be recognized by specific antibodies. Unfortunately, it is generally the case that tumor specific antibodies will not in and of themselves exert sufficient antitumor effects to make them useful in cancer therapy.

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Over the past fifteen years, immunotoxins have shown great promise as a means of selectively targeting cancer cells. Immunotoxins are conjugates of a specific targeting agent typically a tumor-directed antibody or fragment, with a cytotoxic agent, such as a toxin moiety. The targeting agent directs the toxin to, and thereby selectively kills, cells carrying the targeted antigen. Although early immunotoxins suffered from a variety of drawbacks, more recently, stable, long-lived immunotoxins have been developed for the treatment of a variety of malignant diseases. These "second generation" immunotoxins employ deglycosylated ricin A chain to prevent entrapment of the immunotoxin by the liver and hepatotoxicity (Blakey et al., 1987). They employ new crosslinkers which endow the immunotoxins with high *in vivo* stability (Thorpe et al., 1988) and they employ antibodies which have been selected using a rapid indirect screening assay for their ability to form highly potent immunotoxins (Till et al., 1988).

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Immunotoxins have proven highly effective at treating lymphomas and leukemias in mice (Thorpe et al., 1988; Ghetie et al., 1991; Griffin et al., 1988) and in man (Vitetta et al., 1991). Lymphoid neoplasias are particularly amenable to immunotoxin therapy because the tumor cells are relatively accessible to blood-borne immunotoxins; also, it is possible to target normal

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lymphoid antigens because the normal lymphocytes which are killed along with the malignant cells during therapy are rapidly regenerated from progenitors lacking the target antigens. In Phase I trials where patients had large bulky tumor masses, greater than 50% tumor regressions were achieved in approximately 40% of the patients (Vitetta et al., 1991). It is predicted that the efficacy of these immunotoxins in patients with less bulky disease will be even better.

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In contrast with their efficacy in lymphomas, immunotoxins have proved relatively ineffective in the treatment of solid tumors such as carcinomas (Weiner et al., 1989; Byers et al., 1989). The principal reason for this is that solid tumors are generally impermeable to antibody-sized molecules: specific uptake values of less than 0.001% of the injected dose/g of tumor are not uncommon in human studies (Sands et al., 1988; Epenetos et al., 1986). Furthermore, antibodies that enter the tumor mass do not distribute evenly for several reasons. Firstly, the dense packing of tumor cells and fibrous tumor stromas present a formidable physical barrier to macromolecular transport and, combined with the absence of lymphatic drainage, create an elevated interstitial pressure in the tumor core which reduces extravasation and fluid convection (Baxter et al., 1991; Jain, 1990). Secondly, the distribution of blood vessels in most tumors is disorganized and heterogeneous, so some tumor cells are separated from extravasating antibody by large diffusion distances (Jain, 1990). Thirdly, all of the antibody entering the tumor may become adsorbed in perivascular regions by the first tumor cells encountered, leaving none to reach tumor cells at more distant sites (Baxter et al., 1991; Kennel et al., 1991). Finally, antigen-deficient mutants can escape being killed by the immunotoxin and regrow (Thorpe et al., 1988).

Thus, it is quite clear that a significant need exists for the development of novel strategies for the treatment of solid tumors. One approach would be to target cytotoxic agents or coagulants to the vasculature of the tumor rather than to the tumor. Indeed, it has been observed that many existing therapies may already have, as part of their action, a vascular-mediated mechanism of action (Denekamp, 1990). The present inventors propose that this approach offers several advantages over direct targeting of tumor cells. Firstly, the target cells are directly accessible to intravenously administered therapeutic agents, permitting rapid localization of a high percentage of the injected dose (Kennel *et al.*, 1991). Secondly, since each capillary provides oxygen and nutrients for thousands of cells in its surrounding 'cord' of tumor, even limited damage to the tumor vasculature could produce an avalanche of tumor cell death (Denekamp, 1990; Denekamp, 1984). Finally, the outgrowth of mutant endothelial cells lacking the target antigen is unlikely because they are normal cells.

For tumor vascular targeting to succeed, antibodies are required that recognize tumor endothelial cells but not those in normal tissues. Although several antibodies have been raised (Duijvestijn *et al.*, 1987; Hagemeyer *et al.*, 1985; Bruland *et al.*, 1986; Murray *et al.*, 1989; Schlingemann *et al.*, 1985) none has shown a high degree of specificity. The most promising antibodies described thus far are TP-1 and TP-3, which were raised against human osteosarcoma cells. Both antibodies appear to react with the same antigen and, besides reacting with proliferating osteoblasts in normal degenerating bone tissue, cross-react with capillary buds in a number of tumor types and in placenta but not with capillaries in any of the normal adult tissues examined (Bruland *et al.*, 1986). It remains to be seen whether the TP-1/TP-3

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antigen is present on the surface of endothelial cells or whether the antibodies cross-react with gut endothelial cells, as was found with another antibody against proliferating endothelium (Hagemeyer et al., 1985).

- 5 Thus, unfortunately, while vascular targeting presents promising theoretical advantages, no effective strategies incorporating these advantages have been developed.

SUMMARY OF THE INVENTION

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The present invention addresses one or more of the foregoing or other disadvantages in the prior art, by providing a series of novel approaches for the treatment and/or diagnosis (imaging) of vascularized solid tumors.

- 15 The invention rests in a general and overall sense on the use of immunological reagents to target therapeutic or diagnostic agents to tumor-associated vascular endothelial cells, alone or in combination with the direct targeting of tumor cells.

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In the case of diagnostic agents, the constructs will have the ability to provide an image of the tumor vasculature, for example, through magnetic resonance imaging, x-ray imaging, computerized emission tomography and the like.

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- In the case of therapeutic agents, constructs are designed to have a cytotoxic or otherwise anticellular effect against the tumor vasculature, by suppressing the growth or cell division of the vascular endothelial cells. This attack is intended to lead to a tumor-localized vascular collapse, depriving the tumor cells, particularly those tumor cells distal of the vasculature, of oxygen and nutrients, ultimately leading to cell death and tumor necrosis. In animal model systems, the inventors have achieved truly dramatic tumor regressions,
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with some cures being observed in combination therapy with anti-tumor directed therapy.

It is proposed that the various methods and compositions of the invention will be broadly applicable to the treatment or diagnosis of any tumor mass having a vascular endothelial component. Typical vascularized tumors are the solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors to which the present invention is directed include but are not limited to carcinomas of the lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, neuroblastomas, and the like.

The method of the invention includes preparing an antibody that recognizes a cell surface antigen expressed by vascular endothelial cells of the vascularized tumor mass, linking the antibody to the selected agent to form an antibody-agent conjugate, and introducing the antibody-agent conjugate into the bloodstream of an animal, such as a human cancer patient or a test animal in an animal model system. As used however, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgE, F(ab')₂, a univalent fragment such as Fab', Fab, Dab, as well as engineered antibodies such as recombinant antibodies, humanized antibodies, bispecific antibodies, and the like.

The agent that is linked to the antibody will, of course, depend on the ultimate application of the invention. Where the aim is to provide an image of the tumor, one will desire to use an agent that is detectable

upon imaging, such as a paramagnetic, radioactive or
fluorogenic agent. Many agents are known in the art to
be useful for imaging purposes, as are methods for their
attachment to antibodies (see, e.g., U.S. patents
5 5,021,236 and 4,472,509, both incorporated herein by
reference). In the case of paramagnetic ions, one might
mention by way of example ions such as chromium (III),
manganese (II), iron (III), iron (II), cobalt (II),
nickel (II), copper (II), neodymium (III), samarium
10 (III), ytterbium (III), gadolinium (III), vanadium (II),
terbium (III), dysprosium (III), holmium (III) and erbium
(III), with gadolinium being particularly preferred.
Ions useful in other contexts, such as X-ray imaging,
include but are not limited to lanthanum (III), gold
15 (III), lead (II), and especially bismuth (III).
Moreover, in the case of radioactive isotopes for
therapeutic and/or diagnostic application, one might
mention iodine¹³¹, iodine¹²³, technetium^{99m}, indium¹¹¹,
rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰,
20 iodine¹²⁵, or astatine²¹¹ (for a review of the use of
monoclonal antibodies in the diagnosis and therapy of
cancer, see Vaickus et al., 1991).

For certain applications, it is envisioned that
25 pharmacologic agents will serve as useful agents for
attachment to antibodies, particularly cytotoxic or
otherwise anticellular agents having the ability to kill
or suppress the growth or cell division of endothelial
cells. In general, the invention contemplates the use of
30 any pharmacologic agent that can be conjugated to an
antibody and delivered in active form to the targeted
endothelium. Exemplary anticellular agents include
chemotherapeutic agents, radioisotopes as well as
cytotoxins. In the case of chemotherapeutic agents, the
35 inventors propose that agents such as a hormone such as a
steroid; an antimetabolite such as cytosine arabinoside,
fluorouracil, methotrexate or aminopterin; an

anthracycline; mitomycin C; a vinca alkaloid; demecolcine; etoposide; mithramycin; or an antitumor alkylating agent such as chlorambucil or melphalan, will be particularly preferred. Other embodiments may include agents such as a coagulant, a cytokine, growth factor, bacterial endotoxin or the lipid A moiety of bacterial endotoxin. In any event, it is proposed that agents such as these may be successfully conjugated to antibodies in a manner that will allow their targeting, internalization, release or presentation to blood components at the site of the targeted endothelial cells as required using known conjugation technology (see, e.g., Ghose et al., 1983 and Ghose et al., 1987).

In certain preferred embodiments, agents for therapeutic application will include generally a plant-, fungus- or bacteria-derived toxin, such as an A chain toxins, a ribosome inactivating protein, α -sarcin, aspergillin, restirictocin, a ribonuclease, diphtheria toxin or *pseudomonas* exotoxin, to mention just a few examples. The use of toxin-antibody constructs is well known in the art of immunotoxins, as is their attachment to antibodies. Of these, a particularly preferred toxin for attachment to antibodies will be a deglycosylated ricin A chain. Deglycosylated ricin A chain is preferred because of its extreme potency, longer half-life, and because it is economically feasible to manufacture it a clinical grade and scale.

The present invention contemplates two separate and distinct approaches to the targeting of antibodies to the tumor vasculature. The first approach involves the preparation of an antibody having a binding affinity for antigenic markers found, expressed, accessible to binding or otherwise localized on the cell surfaces of tumor-associated vascular endothelium as compared to normal vasculature. Such an antibody is then employed to

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deliver the selected agent to the tumor vasculature. Of course, where a therapeutic as opposed to diagnostic application is envisioned it will be desirable to prepare and employ an antibody having a relatively high degree of tumor vasculature selectivity, which might be expressed as having little or no reactivity with the cell surface of normal endothelial cells as assessed by immunostaining of tissue sections. Of course, with certain agents such as DNA synthesis inhibitors, and, more preferably, antimetabolites, the requirement for selectivity is not as necessary as it would be, for example, with a toxin because a DNA synthesis inhibitor would have relatively little effect on the vascularization of normal tissues because the capillary endothelial cells are not dividing. Further, such a degree of selectivity is not a requirement for imaging purposes since cell death, and hence toxicity, is not the ultimate goal. In the case of diagnostic application, it is proposed that antibodies having a reactivity for the tumor vasculature of at least two-fold higher than for normal endothelial cells, as assessed by immunostaining, will be useful.

This aspect of the invention rests on the proposition that because of their proximity to the tumor itself, tumor-associated vascular endothelial cells are constantly exposed to many tumor-derived products such as cytokines (including lymphokines, monokines, colony-stimulating factors and growth factors), angiogenic factors, and the like, that will serve to selectively elicit the expression of tumor endothelium-specific cell surface markers. In connection with this aspect of the invention, antibodies directed against tumor vasculature are prepared by "mimicking" this phenomenon. Endothelial cells are subjected to tumor-derived products such as might be obtained from tumor-conditioned media. Thus, this method involves generally stimulating endothelial cells with tumor-conditioned medium, employing the

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stimulated endothelial cells as immunogens to prepare a collection of antibody-producing hybridomas, selecting from the collection a hybridoma that produces an antibody that recognizes the tumor-stimulated vascular endothelium to a greater degree than it recognizes non-tumor-stimulated vascular endothelium, and reacts more strongly with tumor-associated endothelial cells in tissue sections than with those in normal adult human tissues, and culturing the hybridoma to provide the antibody.

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Stimulated endothelial cells contemplated to be of use in this regard include, for example, human umbilical vein endothelial cells (HUV), human dermal microvascular endothelial cells (HDEMC), human saphenous vein endothelial cells, human omental fat endothelial cells, human microvascular endothelial cells, human brain capillary endothelial cells, and the like. It is also contemplated that even endothelial cells from another species may be stimulated by tumor-conditioned media and employed as immunogens to generate hybridomas to produce antibodies in accordance herewith, i.e., to produce antibodies which crossreact with tumor-stimulated human vascular endothelial cells.

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As used herein, "tumor-conditioned medium" is defined as a composition or medium, such as a culture medium, which contains one or more tumor-derived cytokines, lymphokines or other effector molecules. Most typically, tumor-conditioned medium is prepared from a culture medium in which selected tumor cells have been grown, and will therefore be enriched in such tumor-derived products. The type of medium is not believed to be particularly important, so long as it at least initially contains appropriate nutrients and conditions to support tumor cell growth. It is also, of course, possible to extract and even separate materials from tumor-conditioned media and employ one or more of the

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extracted products for application to the endothelial cells.

As for the type of tumor used for the preparation of the media, one will, of course, prefer to employ tumors that mimic or resemble the tumor that will ultimately be subject to analysis or treatment using the present invention. Thus, for example, where one envisions the development of a protocol for the treatment of breast cancer, one will desire to employ breast cancer cells such as ZR-75-1, T47D, SKBR3, MDA-MB-231. In the case of colorectal tumors, one may mention by way of example the HT29 carcinoma, as well as DLD-1, HCT116 or even SW48 or SW122. In the case of lung tumors, one may mention by way of example NCI-H69, SW2, NCI H23, NCI H460, NCI H69, or NCI H82. In the case of melanoma, good examples are DX.3, A375, SKMEL-23, HMB-2, MJM, T8 or indeed VUP. In any of the above cases, it is further believed that one may even employ cells produced from the tumor that is to be treated, i.e., cells obtained from a biopsy.

Once prepared, the tumor-conditioned media is then employed to stimulate the appearance of tumor endothelium-specific marker(s) on the cell surfaces of endothelial cells, e.g., by culturing selected endothelial cells in the presence of the tumor-conditioned media (or products derived therefrom). Again, it is proposed that the type of endothelial cell that is employed is not of critical importance, so long as it is generally representative of the endothelium associated with the vasculature of the particular tumor that is ultimately to be treated or diagnosed. The inventors prefer to employ human umbilical vein endothelial cells (HUVE), or human dermal microvascular endothelial cells (HDMEC, Karasek, 1989), in that these cells are of human origin, respond to cytokine growth factors and angiogenic factors and are readily

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obtainable. However, it is proposed that any endothelial cell that is capable of being cultured *in vitro* may be employed in the practice of the invention and nevertheless achieve benefits in accordance with the invention. One may mention by way of example, cells such as EA.hy9.26, ECV304, human saphenous vein endothelial cells, and the like.

Once stimulated using the tumor-derived products, the endothelial cells are then employed as immunogens in the preparation of monoclonal antibodies. The technique for preparing monoclonal antibodies against antigenic cell surface markers is quite straightforward, and may be readily carried out using techniques well known to those of skill in the art, as exemplified by the technique of Kohler & Milstein (1975). Generally speaking, the preparation of monoclonal antibodies using stimulated endothelial cells involves the following procedures. Cells or cell lines derived from human tumors are grown in tissue culture for ≥ 4 days. The tissue culture supernatant ('tumor-conditioned medium') is removed from the tumor cell cultures and added to cultures of HUVEC at a final concentration of 50% (v/v). After 2 days culture the HUVEC are harvested non-enzymatically and $1-2 \times 10^6$ cells injected intraperitoneally into mice. This process is repeated three times at two-weekly intervals, the final immunization being by the intravenous route. Three days later the spleen cells are harvested and fused with SP2/0 myeloma cells by standard protocols (Kohler & Milstein, 1975): Hybridomas producing antibodies with the appropriate reactivity are cloned by limiting dilution.

From the resultant collection of hybridomas, one will then desire to select one of more hybridomas that produce an antibody that recognizes the activated vascular endothelium to a greater extent than it

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recognizes non-activated vascular endothelium. Of course, the ultimate goal is the identification of antibodies having virtually no binding affinity for normal endothelium. However, for imaging purposes this property is not so critical. In any event, one will generally identify suitable antibody-producing hybridomas by screening using, e.g., an ELISA, RIA, IRMA, IIF, or similar immunoassay, against one or more types of tumor-activated endothelial cells. Once candidates have been identified, one will desire to test for the absence of reactivity for non-activated or "normal" endothelium or other normal tissue or cell type. In this manner, hybridomas producing antibodies having an undesirably high level of normal cross-reactivity for the particular application envisioned may be excluded.

The inventors have applied the foregoing technique with some degree of success, in that antibodies having relative specificity for tumor vascular endothelium were successfully prepared and isolated. In this particular example, the inventors employed the HT29 carcinoma to prepare the conditioned medium, which was then employed to stimulate HUVE cells in culture. The resultant HT29-activated HUVE cells were then employed as immunogens in the preparation of a hybridoma bank, which was ELISA-screened using HT29-activated HUVE cells and by immunohistologic analysis of sections of human tumors and normal tissues. From this bank, the inventors have selected antibodies that recognize an antigen that migrates as a doublet upon SDS polyacrylamide gel electrophoresis, each of the doublet bands exhibiting an apparent molecular weight of about 43 kilodaltons.

This 43 K doublet is trypsin sensitive, and it is now known that the antigen is localized to the cell surface of stimulated HUVE cells, and is only minimally present (or immunologically accessible) on the surface of

non-stimulated cells. Furthermore, it has been found that the 43 K antigen is induced when HUVE cells are placed in tissue culture even in the absence of growth factors or serum, and the antigen does not appear to be induced by interleukin 1 (IL-1), tumor necrosis factor (TNF), gamma interferon (IFN- γ), or interleukin 4 (IL-4). The two most preferred monoclonal antibodies prepared by this technique are referred to by the inventors as tumor endothelial cell antibody 4 and 11 (TEC4 and TEC11).

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The second overall general approach presented by the present invention involves the selective elicitation of vascular endothelial antigen targets on the surface of tumor-associated vasculature. This approach targets known endothelial antigens that are present, or inducible, on the cell surface of endothelial cells. The key to this aspect of the invention is the successful manipulation of antigenic expression or surface presentation such that the target antigen is expressed or otherwise available on the surface of tumor associated vasculature and not expressed or otherwise available for binding, or at least to a lesser extent, on the surface of normal endothelium.

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A variety of endothelial cell markers are known that can be employed as inducible targets for the practice of this aspect of the invention, including endothelial-leukocyte adhesion molecule (ELAM-1; Bevilacqua et al., 1987); vascular cell adhesion molecule-1 (VCAM-1; Dustin et al., 1986); intercellular adhesion molecule-1 (ICAM-1; Osborn et al., 1989); the agent for leukocyte adhesion molecule-1 (LAM-1 agent), or even a major histocompatibility complex (MHC) Class II antigen, such as HLA-DR, HLA-DP or HLA-DQ (Collins et al., 1984). Of these, the targeting of ELAM-1 or an MHC Class II antigen will likely be preferred for therapeutic application, with ELAM-1 being particularly preferred, since the

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expression of these antigens will likely be the most direct to promote selectively in tumor-associated endothelium.

5 The targeting of an antigen such as ELAM-1 is the most straightforward since ELAM-1 is not expressed on the surfaces of normal endothelium. ELAM-1 is an adhesion molecule that can be induced on the surface of endothelial cells through the action of cytokines such as
10 IL-1, TNF, lymphotoxin or bacterial endotoxin (Bevilacqua et al., 1987). In the practice of the present invention, the expression of ELAM-1 is selectively induced in tumor endothelium through the use of a bispecific antibody having the ability to cause the selective release of one
15 or more of the foregoing or other appropriate cytokines in the tumor environment, but not elsewhere in the body. This bispecific antibody is designed to cross-link cytokine effector cells, such as cells of monocyte/macrophage lineage, T cells and/or NK cells or
20 mast cells, with tumor cells of the targeted solid tumor mass. This cross-linking is intended to effect a release of cytokine that is localized to the site of cross-linking, i.e., the tumor.

25 Bispecific antibodies useful in the practice of this aspect of the invention, therefore, will have a dual specificity, recognizing a selected tumor cell surface antigen on the one hand, and, on the other hand, recognizing a selected "cytokine activating" antigen on
30 the surface of a selected leukocyte cell type. As used herein, the term "cytokine activating" antigen is intended to refer to any one of the various known molecules on the surfaces of leukocytes that, when bound by an effector molecule such as an antibody or a fragment
35 thereof or a naturally-occurring agent or synthetic analog thereof, be it a soluble factor or membrane-bound counter-receptor on another cell, will promote the

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release of a cytokine by the leukocyte cell. Examples of cytokine activating molecules include CD14 and FcR for IgE, which will activate the release of IL-1 and TNF α ; and CD16, CD2 or CD3 or CD28, which will activate the release of IFN γ and TNF β , respectively.

Once introduced into the bloodstream of an animal bearing a tumor, such a bispecific construct will bind to tumor cells within the tumor, cross-link those tumor cells with, e.g., monocytes/ macrophages that have infiltrated the tumor, and thereafter effect the selective release of cytokine within the tumor. Importantly, however, without cross-linking of the tumor and leukocyte, the bispecific antibody will not effect the release of cytokine. Thus, no cytokine release will occur in parts of the body removed from the tumor and, hence, expression of ELAM-1 will occur only within the tumor endothelium.

A number of useful "cytokine activating" antigens are known, which, when cross-linked with an appropriate bispecific antibody, will result in the release of cytokines by the cross-linked leukocyte. The most preferred target for this purpose is CD14, which is found on the surface of monocytes and macrophages. When CD14 is cross linked it will stimulate the monocyte/ macrophage to release IL-1, and possibly other cytokines, which will, in turn stimulate the appearance of ELAM-1 on nearby vasculature. Other possible targets for cross-linking in connection with ELAM-1 targeting includes FcR for IgE, found on Mast cells; FcR for IgG (CD16), found on NK cells; as well as CD2, CD3 or CD28, found on the surfaces of T cells. Of these, CD14 targeting will be the most preferred due to the relative prevalence of monocyte/macrophage infiltration of solid tumors as opposed to the other leukocyte cell types.

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In that MHC Class II antigens are expressed on "normal" endothelium, their targeting is not quite so straightforward as ELAM-1. However, the present invention takes advantage of the discovery that immunosuppressants such as Cyclosporin A (CsA) have the ability to effectively suppress the expression of Class II molecules in the normal tissues. There are various other cyclosporins related to CsA, including cyclosporins A, B, C, D, G, and the like, which have immunosuppressive action, and will likely also demonstrate an ability to suppress Class II expression. Other agents that might be similarly useful include FK506 and rapamycin.

Thus, the practice of the MHC Class II targeting embodiment requires a pretreatment of the tumor-bearing animal with a dose of CsA or other Class II immunosuppressive agent that is effective to suppress Class II expression. In the case of CsA, this will typically be on the order of about 10 to 30 mg/kg. Once suppressed in normal tissues, Class II antigens can be selectively induced in the tumor endothelium through the use of a bispecific antibody, this one having specificity for the tumor cell as well as an activating antigen found on the surface of helper T cells. Note that in this embodiment, it is necessary that T cells, or NK cells if CD16 is used, be present in the tumor to produce the cytokine intermediate in that Class II antigen expression is achieved using IFN- γ , but is not achieved with the other cytokines. Thus, for the practice of this aspect of the invention, one will desire to select CD2, CD3 or CD28 (most preferably CD28) as the cytokine activating antigen.

An alternative approach to using "cytokine-activating" bispecific antibodies might be to activate the patients peripheral blood leukocytes or tumor-infiltrating lymphocytes *in vitro* (using IL-2 or

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autologous tumor cells for instance), reinfuse them into the patient and then localize them in the tumor with a bispecific antibody against any reliable leukocyte-specific marker, including CD5, CD8, CD11/CD18, CD15, CD32, CD44, CD45 or CD64. In order to selectively localize those leukocytes that had become activated from within a mixed population, it is recommended that the anti-leukocyte arm of the bispecific antibody should recognize a marker restricted to activate cells, such as CD25, CD30, CD54 or CD71. Neither of these approaches is favored as much as the 'cytokine-activating' antibody approach because cross-linking to tumor cells is not a prerequisite for cytokine secretion and thus the resultant induction of cytokine-induced endothelial cell antigens may not be confined to the tumor.

The targeting of the other adhesion molecules, ICAM-1, VCAM-1 and LAM-1 agent, will typically not be preferred for the practice of therapeutic embodiments, in that these targets are constitutively expressed in normal endothelium. Thus, these adhesion molecules will likely only be useful in the context of diagnostic embodiments. Furthermore, it is unlikely that ICAM-1 or VCAM-1 expression by normal endothelial cells would be inhibited *in vivo* by CsA because low levels of expression of both markers are constitutive properties of human endothelial cells (Burrows et al., 1991). However, it may still be possible to utilize one of these molecules in diagnostic or even therapeutic embodiments because their level of expression on the endothelial cell surface is increased 10-50 fold by cytokines. As a consequence, there may be a therapeutic or diagnostic 'window' enabling use of anti-ICAM-1 or anti-VCAM-1 conjugates in an analogous way to the proven clinical utility of some antibodies against 'tumor-associated' antigens whose expression differs quantitatively but not qualitatively from normal tissues.

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The tumor antigen recognized by the bispecific antibodies employed in the practice of the present invention will be one that is located on the cell surfaces of the tumor being targeted. A large number of solid tumor-associated antigens have now been described in the scientific literature, and the preparation and use of antibodies are well within the skill of the art (see, e.g., Table II hereinbelow). Of course, the tumor antigen that is ultimately selected will depend on the particular tumor to be targeted. Most cell surface tumor targets will only be suitable for imaging purposes, while some will be suitable for therapeutic application. For therapeutic application, preferred tumor antigens will be TAG 72 or the HER-2 proto-oncogene protein, which are selectively found on the surfaces of many breast, lung and colorectal cancers (Thor et al., 1986; Colcher et al., 1987; Shepard et al., 1991). Other targets that will be particularly preferred include milk mucin core protein, human milk fat globule (Miotti et al., 1985; Burchell et al., 1983) and even the high Mr melanoma antigens recognized by the antibody 9.2.27 (Reisfeld et al., 1982).

In still further embodiments, the inventors contemplate an alternative approach for suppressing the expression of Class II molecules, and selectively eliciting Class II molecule expression in the locale of the tumor. This embodiment takes advantage of the fact that the expression of Class II molecules can be effectively inhibited by suppressing IFN- γ production by T-cells, e.g., through use of an anti-CD4 antibody (Street et al., 1989). Thus, in this embodiment, one will desire to pretreat with a dose of anti-CD4 that is effective to suppress IFN- γ production and thereby suppress the expression of Class II molecules (for example, on the order of 4 to 10 mg/kg). After Class II expression is suppressed, one will then prepare and

introduce into the bloodstream an IFN- γ -producing T-cell clone (e.g., T_H1 or CTL) specific for an antigen expressed on the surface of the tumor cells.

5 A preferred means of producing the IFN- γ -producing T-cell clone is by a method that includes removing a portion of the tumor mass from the patient, extracting tumor infiltrating leukocytes from the tumor, and expanding the tumor infiltrating leukocytes *in vitro* to
10 provide the IFN- γ producing clone. This clone will necessarily be immunologically compatible with the patient, and therefore should be well tolerated by the patient. It is proposed that particular benefits will be achieved by further selecting a high IFN- γ producing
15 T-cell clone from the expanded leukocytes by determining the cytokine secretion pattern of each individual clone every 14 days. To this end, rested clones will be mitogenically or antigenically-stimulated for 24 hours and their culture supernatants assayed by a specific
20 sandwich ELISA technique (Cherwinski et al., 1989) for the presence of IL-2, IFN- γ , IL-4, IL-5 and IL-10. Those clones secreting high levels of IL-2 and IFN- γ , the characteristic cytokine secretion pattern of T_H1 clones, will be selected. Tumor specificity will be confirmed
25 using proliferation assays. Furthermore, one will prefer to employ as the anti-CD4 antibody an anti-CD4 Fab, because it will be eliminated from the body within 24 hours after injection and so will not cause suppression of the tumor recognizing T cell clones that are
30 subsequently administered. The preparation of T-cell clones having tumor specificity is generally known in the art, as exemplified by the production and characterization of T cell clones from lymphocytes infiltrating solid melanoma tumors (Maeda et al., 1991).

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The invention contemplates that still further advantages will be realized through combination regimens

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wherein both the tumor endothelial vasculature and the tumor itself are targeted. Combination regimens may thus include targeting of the tumor directly with either conventional antitumor therapy, such as with radiotherapy or chemotherapy, or through the use of a second immunological reagent such as an antitumor immunotoxin. In fact, dramatic, synergistic antitumor effects were seen by the inventors when solid tumors were targeted with both an antitumor endothelial cell immunotoxin and an antitumor cell immunotoxin. Such combination therapy is founded theoretically on 1) the use of the endothelial-directed immunotoxin to kill those tumor cells that depend upon vascular oxygen and nutrients, and 2) the use of the tumor-directed immunotoxin to kill those tumor cells that may have an alternate source of oxygen and nutrients (i.e., those tumor cells lining the vasculature and those forming the outer boundary of the tumor mass). Thus, it is proposed that particular advantages will be realized through the targeting of agents both to tumor cell targets as well as to tumor endothelial cell targets.

The invention further contemplates the selected combinations of agents particularly adapted for use in connection with the methods of the present invention, defined as including a first pharmaceutical composition which includes a bispecific antibody recognizing an activating antigen on the cell surface of a leukocyte cell and a tumor antigen on the cell surface of tumor cells of a vascularized solid tumor, together with a second pharmaceutical composition comprising a second antibody or fragment thereof linked to a selected therapeutic or diagnostic agent that recognizes the induced endothelial antigen. In accordance with one aspect of the invention, these agents may be conveniently packaged together, being suitably aliquoted into separate

containers, and the separate containers dispensed in a single package.

In particular embodiments, the activating antigen induced by the bispecific antibody will be CD2, CD3, CD14, CD16, FcR for IgE, CD28 or the T-cell receptor antigen, as may be the case. However, preferably, the bispecific antibody will recognize CD14, and induce the expression of IL-1 by monocyte/macrophage cells in the tumor, or recognize CD28 and induce the expression of IFN- γ by T-cells in the tumor. Where IL-1 is the cytokine intermediate, the second antibody will preferably be one that recognizes ELAM-1, since this adhesion molecule will be induced on the surface of endothelial cells by IL-1. In contrast, where IFN- γ is the intermediate, the second antibody will preferably be one that recognizes an MHC Class II antigen. In the later case, one might desire to include with the combination a third pharmaceutical composition comprising one of the cyclosporins, or another immunosuppressive agent useful for suppressing Class II expression.

Furthermore, in that the invention contemplates combination regimens as discussed above, particular embodiments of the invention will involve the inclusion of a third pharmaceutical composition comprising an antitumor antibody conjugated to a selected agent, such as an anti-tumor immunotoxin. In these embodiments, particularly preferred will be the targeting of tumor antigens such as p185^{HER2}, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA), the high Mr melanoma antigens recognized by the 9.2.27 antibody, or the ovarian-associated antigens recognized by OV-TL3 or MOV18. These same antigens will also be preferred as the target for the bispecific antibody. Of course, where such a bispecific antibody is employed in combination with an antitumor antibody, it may be desirable to target

different tumor antigens with the bispecific and antitumor antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

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Fig. 1. Induction of I-E^k on SVEC cells by IFN-γ in C1300(Muγ)-conditioned medium. In (a), SVEC cells were cultured for 72 hours in regular medium (-----), r.IFN-γ (.....) or r.IFN-γ plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured by M5/114 antibody binding by indirect immunofluorescence using the FACS. Other cultures were treated with r.IFN-γ and stained with an isotype-matched control antibody (- - - -). In (b), SVEC cells were cultured for 72 hours in C1300-conditioned medium (-----), C1300(Muγ)-conditioned medium (.....) or C1300(Muγ)-conditioned medium plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured as in (a). Other cultures were treated with C1300(Muγ)-conditioned medium and stained with an isotype-matched control antibody (- - -).

Fig. 2. Expression of I-E^k and H-2K^k by pure and mixed populations of C1300 and C1300(Muγ) cells. C1300 cells (. . . .), C1300(Muγ) cells (- - -), a mixture of C1300 and C1300(Muγ) cells in the ratio 7:3 cocultured in vitro (.....) or cells recovered from a mixed subcutaneous tumor in a BALB/c nu/nu mouse (-----) were stained with (a) anti-I-E^k antibody or (b) anti-H-2K^k antibody by indirect immunofluorescence using the FACS. No staining of any tumor cell population was seen with the isotype-matched control antibodies.

Fig. 3. Tumorigenicity, growth, and tumor endothelial cell Ia^d expression in pure and mixed subcutaneous C1300 and C1300(Muγ) tumors. BALB/c nu/nu mice were injected with a total of 2x10⁷ tumor cells in

which the ratios of C1300: C1300(Mu γ) cells were 10:0 (Δ), 9:1 (O), 8:2 (\bullet), 7:3 (\diamond), 5:5 (\blacksquare), 3:7 (\square) or 0:10 (Δ). The vertical axis shows the mean diameter of the tumors at various times after injection. Also shown are the percentage of animals in each group which developed tumors. The proportion of Ia^d-positive vascular endothelial cells was categorized as follows: +, 75-100%; +/-, 25-75%; -, 0-5%; n.d., not determined because no intact blood vessels were visible. Standard deviations were < 15% of mean diameters and are not shown.

Fig. 4. Comparison of killing activity of anti-Class II immunotoxin (M5/114 dgA) against unstimulated SVEC mouse endothelial cells (Fig. 4A) and those stimulated with conditioned medium from the IFN- γ -secreting tumor C1300(Mu- γ). The data shown are for treatment of cells with varying concentrations of ricin (\square); M5/114dgA (\bullet); and the control immunotoxin CAMPATH-2dgA (O).

Fig. 5. This figure also shows the killing of SVEC cells under various conditions by the anti-Class II immunotoxin, M5/114dgA. The data shown are for treatment of cells with varying concentrations of the immunotoxin following treatment with IFN- γ TCM (O); C1300 TCM (Δ); C1300(Mu- γ) TCM (\blacksquare); and C1300(Mu- γ) treated with anti-IFN- γ (\square).

Fig. 6. The upper figure, Fig. 6A, shows a comparison of killing activity of an anti-Class I (antitumor) immunotoxin (11-4.1-dgA, which recognized H-2K^k) and an anti-Class II (anti-tumor endothelial cell) immunotoxin (M5/114-dgA) against a 70:30 mixed population of C1300 and C1300(Mu- γ) cells. The lower figure, Fig. 6B, shows killing of cells freshly recovered from subcutaneous tumors in mice. Both figures show data obtained through treatment of the cells with ricin ();

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the 11-4.1-dgA immunotoxin (O); the M5/114-dgA immunotoxin (■) and a control immunotoxin (□).

Fig. 7. This figure shows a comparison of the killing of pure populations of C1300 (●) and C1300(Mu-γ) (O) by the antitumor cell immunotoxin, 11-4.1-dgA. Also shown are 70:30 mixed populations mixed *in vitro* or *in vivo* (i.e., recovered from S/C tumors). Also shown are controls, including ricin (Δ) and a control immunotoxin (Δ).

Fig. 8. This figure shows the *in vivo* antitumor effects of the anti-endothelial cell immunotoxin, M5/114-dgA, at various doses, including 20 μg (O) and 40 μg (■). These studies involved the administration of the immunotoxin intravenously 14 days after injection of tumor cells. Controls included the use of a control immunotoxin, CAMPATH-2-dgA (Δ) and PBS + BSA (Δ).

Figs. 9 and 10. These figures are histological analyses of 1.2 cm H&E-stained tumor sections 72 hours after treatment with 20 μg (Fig. 9) or 100 μg (Fig. 10) of the anti-Class II immunotoxin, M5/114-dgA.

Fig. 11. This figure is a representation of the appearance of a solid tumor 48-72 hours after intravenous immunotoxin treatment, and compares the effect achieved with anti-tumor immunotoxin, to that achieved with anti-endothelial cell immunotoxin therapy.

Fig. 12. This figure shows the antitumor effects of single and combined treatments with anti-Class I and anti-Class II immunotoxins in SCID mice bearing large solid C1300(Mu-γ) tumors. SCID mice bearing 1.0-1.3 cm diameter tumors were injected intravenously 14 days after tumor inoculation with 20μg of Class II immunotoxin (O), 100 μg Class I immunotoxin (●), or diluent alone (Δ).

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Other animals received the anti-Class II immunotoxin followed by two days later by the anti-Class I immunotoxin (■), or vice versa (□). Tumor size was measured at regular intervals and is expressed as mean tumor diameter +/- SEM. Each treatment group consisted of 4-8 mice.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

10 A. INTRODUCTION

A solution to the problem of poor penetration of antibodies into solid tumors is to attack the endothelial cells (EC) lining the blood vessels in the tumor. This approach offers several advantages over direct targeting of tumor cells. Firstly, the target cells are directly accessible to intravenously administered therapeutic agents, permitting rapid localization of a high percentage of the injected dose (Kennel, et al., 1991). Secondly, since each capillary provides oxygen and nutrients for thousands of cells in its surrounding 'cord' of tumor, even limited damage to the tumor vasculature could produce an avalanche of tumor cell death (Denekamp, 1984). Finally, the outgrowth of mutant endothelial cells lacking the target antigen is unlikely because they are normal and not neoplastic cells.

For tumor vascular targeting to succeed, antibodies are required that recognize tumor endothelial cells but not those in normal tissues. Numerous differences between tumor blood vessels and normal ones have been documented (reviewed in Denekamp, 1990; Dvorak et al., 1991; and Jain, 1988) which suggested to the inventors that antigenic differences might exist. For example, tumors elaborate angiogenic factors (Kandel et al., 1991; Folkman, 1985a;b) and cytokines (Burrows et al., 1991; Ruco et al., 1990; Borden et al., 1990) which alter the

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behavior and phenotype of local endothelial cells. Vascular endothelial cells in tumors proliferate at a rate 30-fold greater than those in miscellaneous normal tissues (Denekamp & Hobson, 1982), suggesting that

5 proliferation-linked determinants could serve as markers for tumor vascular endothelial cells. Nevertheless, despite fairly intensive efforts in several laboratories (Duijvestijn et al., 1987, Blakey et al., 1987a; 1987b; Knowles & Thorpe, 1987), antibodies have not yet been

10 obtained which clearly distinguish tumor from normal vasculature.

The present inventors have developed a variety of strategies for specifically targeting antibodies to tumor

15 vasculature that address the shortcomings in the prior approaches. One strategy for vascular targeting presented by the invention involves a novel approach to the identification of tumor vascular antigens. This approach employs the induction of specific antigens on

20 vascular endothelial cells using tumor derived products, such as might be obtained from tumor-conditioned cell culture medium. The conditioned media, which undoubtedly includes numerous cytokines, growth factors and tumor-specific products, mimics the solid tumor vascular

25 environment and thereby promotes the appearance of specific tumor vascular antigen markers. This allows for the targeting of the tumor vasculature with an antibody linked to a selected therapeutic or diagnostic agent.

30 A second approach involves the selective induction of MHC Class II molecules on the surfaces of tumor-associated endothelium which can then serve as endothelial cell targets. This approach, however, requires that MHC Class II expression be effectively

35 inhibited in normal tissues. It is known that CSA and related immun suppressants have this capability via inhibition of T cell activation, and can therefore be

employed to pr treat the patient or animal to inhibit Class II expression. Alternatively, it is proposed that inhibition of Class II expression can be achieved using anti-CD4 in that CD4 directed antibodies are known to additionally suppress T cell function (Street et al., 1989). Then, Class II targets are selectively induced in the tumor-associated vascular endothelium through a locally released cytokine intermediate (IFN- γ).

10 A related but distinct approach to targeting tumor vasculature involves the selective elicitation of an endothelial marker in the tumor vascular endothelium. The inventors propose to selectively induce the expression of one or more of the various endothelial
15 adhesion molecules, such as ELAM-1, VCAM-1, ICAM-1, LAM-1 Agent etc., and targeting one of these antigens with an appropriate antibody. Of these, ELAM-1 is the preferred target in that it is quite clear that this antigen is not expressed in normal endothelial vasculature (Cotran et
20 al., 1986). The other adhesion molecules appear to be expressed to varying degrees in other normal tissues, generally in lymphoid organs and on endothelium, making their targeting perhaps appropriate only in diagnostic embodiments.

25 In either case, the key is the use of a bispecific "cytokine-inducing" antibody that will selectively induce the release of the appropriate cytokine in the locale of the tumor. This specifically localized release of
30 cytokine is achieved through a bispecific antibody having the ability to "cross-link" cytokine-producing leukocytes to cells of the tumor mass. The preparation and use of bispecific antibodies such as these is predicated in part on the fact that cross-linking antibodies recognizing
35 CD3, CD14, CD16 and CD28 have previously been shown to elicit cytokine production selectively upon cross-linking with the second antigen (Qian et al., 1991). In the

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context of the present invention, since only successfully
tumor cell-crosslinked leukocytes will be activated to
release the cytokine, cytokine release will be restricted
to the locale of the tumor. Thus, expression of ELAM-1
5 will be similarly limited to the endothelium of the tumor
vasculature.

An overview of various exemplary inducible vascular
endothelial targets, as well as the mechanisms for their
10 induction, is set forth in Table I. This Table lists
various potential endothelial cell targets, such as ELAM-
1, VCAM-1, etc., the inducing intermediate cytokine, such
as IL-1, IFN- γ , etc., and the leukocyte cell type and
associated cytokine activating molecule whose targeting
15 will result in the release of the cytokine. Thus, for
example, a bispecific antibody targeted to an appropriate
solid tumor antigen and CD14, will promote the release of
IL-1 by tumor-localized monocytes and macrophages,
resulting in the selective expression of the various
20 adhesion molecules in the tumor vascular endothelium.
Alternatively, the bispecific antibody may be targeted to
FcR for IgE, FcR for IgG (CD16), CD2, CD3, or CD28, and
achieve a similar result, with the cytokine intermediate
and cytokine-producing leukocyte being different or the
25 same.

TABLE I: POSSIBLE INDUCIBLE VASCULAR TARGETS

INDUCIBLE ENDOTHELIAL CELL MOLECULES	ACRO- NYM	SUBTYPES/ ALIASES (MOLECULAR FAMILY)	INDUCING CYTOKINES	LEUKOCYTES WHICH PRODUCE THOSE CYTOKINES	LEUKOCYTE MOLECULES WHICH, WHEN CROSS- LINKED, BY MONOCLONAL ANTIBODIES ACTIVATE THE CELLS TO PRODUCE CYTOKINES
Endothelial- Leukocyte Adhesion Molecule-1	ELAM-1	-- (Selectin)	IL-1, TNF- α , (TNF- β) (Bacterial Endotoxin)	monocytes	CD14
				macrophages	CD14
				mast cells	FCR for IgE
Vascular Cell Adhesion Molecule-1	VCAM-1	Inducible Cell Adhesion Molecule-110 (INCAM-110) (Immunoglobulin Family)	(Bacterial Endotoxin) IL-1, TNF- α	monocytes	CD14
				macrophages	CD14
				mast cells	FCR for IgE
			TNF- β , IL- 4	helper T cells	CD2, CD3, CD28
Intercellular Adhesion Molecule-1	ICAM-1	-- (Immunoglobulin Family)	TNF	NK cells	FCR for IgG (CD16)
			IL-1, TNF α (Bacterial Endotoxin)	monocytes	CD14
				macrophages	CD15
				mast cells	FCR for IgE
			TNF- β , IFN γ	T helper	CD2, CD3, CD28
				NK cells	FCR for IgG (CD16)

TABLE I continued: POSSIBLE INDUCIBLE VASCULAR TARGETS

INDUCIBLE ENDOTHELIAL CELL MOLECULES	ACRO- NYM	SUBTYPES/ ALIASES (MOLECULAR FAMILY)	INDUCING CYTOKINES	LEUKOCYTES WHICH PRODUCE THOSE CYTOKINES	LEUKOCYTE MOLECULES WHICH, WHEN CROSS- LINKED BY MONOCLONAL ANTIBODIES ACTIVATE THE CELLS TO PRODUCE CYTOKINES
The Agent for Leukocyte Adhesion Molecule-1	LAM-1 Agent	MEL-14 Agent (Mouse)	IL-1, TNF α (Bacterial Endotoxin)	monocytes macrophages mast cells	CD14 CD14 FcR for IgE
Major Histocompatibility Complex Class II Antigen	MHC Class II	HLA-DR HLA-DP - Human HLA-DQ I-A - Mouse I-E	IFN- γ	helper T cells NK cells	CD2, CD3, CD28 FcR for IgG (CD16)

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As pointed out, the distinction between the selective activation of ELAM-1 and the MHC Class II molecules rests on the fact that ELAM-1 is not normally expressed in normal epithelium, whereas Class II molecules are normally expressed in normal endothelium. Thus, when one seeks to target MHC Class II antigens, it will be important to first inhibit their expression in normal tissues using CsA or a similar immunosuppressant agent having the ability to suppress MHC Class II expression. Then, MHC Class II molecules can be selectively induced in the tumor vasculature using, e.g., a bispecific antibody against a solid tumor antigen that activates T_H1 cells in the tumor in a CsA-independent fashion, such as CD28. Such an antibody will trigger the release of IFN- γ which, in turn, will result in the selective expression of Class II molecules in the tumor vasculature.

An alternative approach that avoids both the use of CsA and a bispecific activating antibody involves the use of anti-CD4 to suppress IFN- γ production, followed by introduction of an IFN- γ -producing T-cell clone (e.g., T_H1 or cytotoxic T-lymphocytes (CTLs)) that is specific for a selected tumor antigen. In this embodiment, the T-cell clone itself localizes to the tumor mass due to its antigen recognition capability, and only upon such recognition will the T-cell clone release IFN- γ . In this manner, cytokine release is again restricted to the tumor, thus limiting the expression of Class II molecules to the tumor vasculature.

T lymphocytes from the peripheral blood (Mazzocchi et al., 1990) or within the tumor mass (Fox et al., 1990) will be isolated by collagenase digestion where necessary, and density gradient centrifugation followed by depletion of other leukocyte subsets by treatment with specific antibodies and complement. In addition, CD4⁺ or

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CD8⁺ T cell subsets may be further isolated by treatment with anti-CD8 or anti-CD4 and complement, respectively. The remaining cells will be plated at limiting dilution numbers in 96-well (round bottom) plates, in the presence
5 of 2×10^5 irradiated (2500 rad) tumor cells per well. Irradiated syngeneic lymphocytes (2×10^5 per well) and interleukin-2 (10 U/ml) will also be included. Generally, clones can be identified after 14 days of *in vitro* culture. The cytokine secretion pattern of each
10 individual clone will be determined every 14 days. To this end, rested clones will be mitogenically or antigenically-stimulated for 24 hours and their culture supernatants assayed for the presence of IL-2, IFN- γ , IL-4, IL-5 and IL-10. Those clones secreting high levels
15 of IL-2 and IFN- γ , the characteristic cytokine secretion pattern of T_H1 clones, will be selected. Tumor specificity will be confirmed utilizing proliferation assays.

20 Supernatants obtained after 24 hour mitogen or antigen-stimulation will be analyzed in the following cytokine assays: IL-2, IFN- γ , IL-4, IL-5 and IL-10. The levels of IL-2 and IL-4 will be assayed using the HT-2 bioassay in the presence of either anti-IL-2, anti-IL-4
25 antibodies or both. The remaining cytokines will be assayed using specific two-site sandwich ELISAs (Cherwinski et al., 1989). Cytokines in the unknown samples will be quantitated by comparison with standard curves, by using either linear or four-parameter curve-
30 fitting programs.

A few generalizations can be made as to which approach would be the more appropriate for a given solid tumor type. Generally speaking, the more "immunogenic"
35 tumors would be more suitable for the MHC Class II approach involving, e.g., the cross-linking of T-cells in the tumor through an anti-CD28/anti-tumor bispecific

antibody, because these tumors are more likely to be infiltrated by T cells, a prerequisite. Examples of immunogenic solid tumors include renal carcinomas, melanomas, a minority of breast and colon cancers, as well as possibly pancreatic, gastric, liver, lung and glial tumor cancers. These tumors are referred to as "immunogenic" because there is evidence that they elicit immune responses in the host and they have been found to be amenable to cellular immunotherapy (Yamaue et al., 1990).

In the case of melanomas and large bowel cancers, the most preferred antibodies for use in these instances would be B72.3 (anti-TAG-72) and PRSC5/PR4C2 (anti-Lewis a) or 9.2.27 (anti-high Mr melanoma antigen).

For the majority of solid tumors of all origins, an anti-CD14 approach that employs a macrophage/monocyte intermediate would be more suitable. This is because most tumors are rich in macrophages. Examples of macrophage-rich tumors include most breast, colon and lung carcinomas. Examples of preferred anti-tumor antibodies for use in these instances would be anti-HER-2, B72.3, SM-3, HMFG-2, and SWA11 (Smith et al., 1989).

The inventors have recently developed a model system in the mouse in which to demonstrate and investigate immunotoxin-mediated targeting of vascular endothelial cells in solid tumors. A neuroblastoma transfected with the murine interferon- γ (IFN- γ) is grown in SCID or BALB/c nude mice reared under germ-free conditions. The IFN- γ secreted by the tumor cells induces the expression of MHC Class II antigens on the vascular endothelial cells in the tumor. Class II antigens are absent from the vasculature of normal tissues in germ-free SCID and nude mice although they are present on certain non-life-

sustaining normal cells (such as on B lymphocytes and monocytes) and some epithelial cells.

When mice with large (1.2 cm diameter) tumors were injected with anti-Class II-ricin A chain immunotoxin, dramatic anti-tumor effects were observed. Histological examination of tumors taken from mice at various times after injecting the immunotoxin revealed that vascular endothelial cell degeneration was the first discernable event followed by platelet deposition on the injured vessels and coagulation of the tumor blood supply. This was followed by extensive tumor cell degeneration which occurred within 24 hours after injection of the immunotoxin. By 72 hours, no viable tumor cells remained apart from a few cells on the edge of the tumor where it penetrated into the normal tissues. These surviving tumor cells could be killed by administering an immunotoxin directed against the tumor cells themselves, resulting in lasting complete tumor regressions in a third of the animals.

These background studies have demonstrated the feasibility of targeting tumor vasculature through targeting of MHC Class II or adhesion molecules such as ELAM-1.

B. MHC Class II

Class II antigens are expressed on vascular endothelial cells in most normal tissues in several species, including man (9). Studies *in vitro* (Collins et al., 1984; Daar et al., 1984; O'Connell et al., 1990) and *in vivo* (Groenewegen et al., 1985) have shown that the expression of Class II antigens by vascular endothelial cells requires the continuous presence of IFN- γ which is elaborated by T_{H1} cells and, to a lesser extent, by NK cells and $CD8^+$ T cells. As shown in the dog (Groenewegen

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et al., 1985) and as confirmed by the inventors in normal mice, Class II expression through the vasculature is abolished when CsA is administered. The CsA acts by preventing the activation of T cells and NK cells
5 (Groenewegen et al., 1985; DeFranco, 1991), thereby reducing the basal levels of IFN- γ below those needed to maintain Class II expression on endothelium.

A strategy for confining Class II expression to
10 tumor vasculature is to suppress IFN- γ production throughout the animal by administering CsA and then to induce IFN- γ production specifically in the tumor by targeting a CsA-resistant T cell activator to the tumor. A
15 bispecific (Fab'-Fab') antibody having one arm directed against a tumor antigen and the other arm directed against CD28 should localize in the tumor and then crosslink CD28 antigens on T cells in the tumor. Crosslinking of CD28, combined with a second signal
(provided, for example, by IL-1 which is commonly
20 secreted by tumor cells (Burrows et al., 1991; Ruco et al., 1990)) has been shown to activate T cells through a CA^{2+} -independent non-CsA-inhibitable pathway (Hess et al., 1991; June et al., 1987; Bjorndahl et al., 1989). The T cells that should be activated in the tumor are
25 those adjacent to the vasculature since this is the region most accessible to cells and is also where the bispecific antibody will be most concentrated. The activated T cells should then secrete IFN- γ which induces Class II antigens on the adjacent tumor vasculature.

30

MHC Class II antigens are not unique to vascular endothelial cells. They are expressed constitutively on B cells, activated T cells, cells of monocyte/macrophage lineage and on certain epithelial cells both in mice
35 (Hammerling, 1976) and in man (Daar et al., 1984). It would therefore be anticipated that damage to these normal tissues would result if anti-Class II immunotoxin

-38-

were to be administered. However this presumption is not correct, at least in mice. Anti-Class II immunotoxins administered intravenously to germ-free SCID or BALB/c nude mice are no more toxic to the mice than are immunotoxins having no reactivity with mouse tissues. There are a number of possible explanations for this surprising result. First, anti-Class II antibodies injected intravenously do not appear to reach the epithelial cells or the monocytes/macrophages in organs other than the liver and spleen. Presumably this is because the vascular endothelium in most organs is tight, not fenestrated as it is in the liver and spleen, and so the antibodies must diffuse across basement membranes to reach the Class II-positive cells.

Secondly, hepatic Kupffer cells and probably other cells of monocyte/macrophage lineage are not killed by the anti-Class II immunotoxin even though it binds to them. No morphological changes in the Kupffer cells are visible even several days after administration of the immunotoxin. This is probably because cells of monocyte/macrophage lineage are generally resistant to immunotoxin-mediated killing (Engert et al., 1991). Cells of monocyte/macrophage lineage appear to bind and internalize immunotoxins but route them to the lysosomes where they are destroyed, unlike other cell types which route immunotoxins to the trans-Golgi region or the E.R. which are thought to be site(s) from which ricin A chain enters the cytosol (Van Deurs et al., 1986; Van Deurs et al., 1988).

Finally, there were little morphological evidence of splenic damage despite the fact that the immunotoxin bound to the B cells and that the cells are sensitive to anti-Class II immunotoxins (Lowe et al., 1986; Wu et al., 1990). It is possible that the B cells were killed, but, being metabolically inactive, they degenerated very

-39-

slowly. In any event, B cell elimination is unlikely to be a significant problem in mice or in man because the cells would be replenished from Class II negative progenitors (Lowe et al., 1986); indeed, in B lymphoma patients and normal monkeys treated with anti-B cell immunotoxins, B cell killing definitely occurs but causes no obvious harm (Vitetta et al., 1991).

C. ELAM-1

10

In contrast to Class II, ELAM-1 is not found on the vasculature of normal tissues in humans and is absent from any other cell types (Cotran et al., 1986). It is induced on vascular endothelial cells by IL-1 and TNF but not by IFN- γ (Wu et al., 1990). Its induction is rapid, peaking at 4-6 hours and, thereafter, it is rapidly lost, being hardly detectable by 24 hours (Bevilacqua et al., 1987).

20 With ELAM-1, the strategy is to induce its expression selectively on tumor vasculature using a bispecific antibody that will home to the tumor and activate monocytes/macrophages within the tumor. The bispecific antibody will have one Fab' arm directed against a tumor antigen and the other directed against CD14 (the LPS receptor). After localizing in the tumor, the bispecific antibody should crosslink CD14 receptors on monocytes and the macrophages within the tumor. This should result in powerful activation of these cells (Schutt et al., 1988; Chen et al., 1990) and the production of IL-1 and TNF which will induce ELAM-1 on tumor vascular endothelial cells.

35

D. PREPARATION OF ANTIBODIES

The origin or derivation of the antibody or antibody fragment (e.g., Fab', Fab or F(ab')₂) is not believed to

-40-

be particularly crucial to the practice of the invention, so long as the antibody or fragment that is actually employed for the preparation of bispecific antibodies otherwise exhibit the desired activating or binding properties. Thus, where monoclonal antibodies are employed, they may be of human, murine, monkey, rat, hamster, chicken or even rabbit origin. The invention contemplates that the use of human antibodies, "humanized" or chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, single domain antibodies (e.g., DABs), Fv domains, as well as recombinant antibodies and fragments thereof. Of course, due to the ease of preparation and ready availability of reagents, murine monoclonal antibodies will typically be preferred.

In general, the preparation of bispecific antibodies is also well known in the art, as exemplified by Glennie et al. (1987), as is their use in the activation of leukocytes to release cytokines (Qian et al., 1991). Bispecific antibodies have even been employed clinically, for example, to treat cancer patients (Bauer et al., 1991). Generally speaking, in the context of the present invention the most preferred method for their preparation involves the separate preparation of antibodies having specificity for the targeted tumor cell antigen, on the one hand, and the targeted activating molecule on the other. While numerous methods are known in the art for the preparation of bispecific antibodies, the Glennie et al. method preferred by the inventors involves the preparation of peptic $F(ab'\gamma)_2$ fragments from the two chosen antibodies (e.g., an antitumor antibody and an anti-CD14 or anti-CD28 antibody), followed by reduction of each to provide separate $Fab'\gamma_{SH}$ fragments. The SH groups on one of the two partners to be coupled are then alkylated with a cross-linking reagent such as o-phenylenedimaleimide to provide free maleimide groups

on one partner. This partner may then be conjugated to the other by means of a thioether linkage, to give the desired $F(ab'\gamma)_2$ heteroconjugate.

5 While, due to ease of preparation and high yield and reproducibility, the Glennie et al. (1987) method is preferred for the preparation of bispecific antibodies, there are of course numerous other approaches that can be employed and that are envisioned by the inventors. For
10 example, other techniques are known wherein crosslinking with SPDP or protein A is carried out, or a trispecific construct is prepared (Titus et al., 1987; Tutt et al., 1991). Furthermore, recombinant technology is now available for the preparation of antibodies in general,
15 allowing the preparation of recombinant antibody genes encoding an antibody having the desired dual specificity (Van Duk et al., 1989). Thus, after selecting the monoclonal antibodies having the most preferred binding and activation characteristics, the respective genes for
20 these antibodies can be isolated, e.g., by immunological screening of a phage expression library (Oi & Morrison, 1986; Winter & Milstein, 1991). Then, through rearrangement of Fab coding domains, the appropriate chimeric construct can be readily obtained.

25 The preparation of starting antibodies against the various cytokine activating molecules is also well known in the art. For example, the preparation and use of anti-CD14 and anti-CD28 monoclonal antibodies having the
30 ability to induce cytokine production by leukocytes has now been described by several laboratories (reviewed in Schutt et al., 1988; Chen et al., 1990, and June et al., 1990, respectively). Moreover, the preparation of monoclonal antibodies that will stimulate leukocyte
35 release of cytokines through other mechanisms and other activating antigens is also known (Clark et al., 1986; Geppert et al., 1990).

Similarly, there is a very broad array of antibodies known in the art that have immunological specificity for the cell surface of virtually any solid tumor type, as a vast number of solid tumor assorted antigens have been identified (see, e.g., Table II). Methods for the development of antibodies that are "custom-tailored" to the patient's tumor are likewise known (Stevenson et al., 1990). Of course, not all antibodies will have sufficient selectivity, specificity, affinity and toxin-delivering capability to be of use in the practice of the invention. These properties can be readily evaluated using conventional immunological screening methodology.

TABLE II
MARKER ANTIGENS OF SOLID TUMORS AND CORRESPONDING MONOCLONAL ANTIBODIES

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
A: Gynecological			
GY	'CA 125' > 200 kD mucin GP	OC 125	Kabawat et al., Int. J. Gynecol. Pathol, 4:265, 1983; Szymendera, Tumour Biology, 7:333, 1986
ovarian	80 Kd GP	OC 133	Masuko et al, Cancer Res., 44:2813, 1984
ovarian	'SGA' 360 Kd GP	OMI	de Krestet et al., Int. J. Cancer, 37:705, 1986
ovarian	High M_r mucin	Mo v1	Miotti et al, Cancer Res., 65:826, 1985
ovarian	High M_r mucin/ glycolipid	Mo v2	Miotti et al, Cancer Res., 65:826, 1985
ovarian	NS	3C2	Tsuji et al., Cancer Res., 45:2358, 1985
ovarian	NS	4C7	Tsuji et al., Cancer Res., 45:2358, 1985
ovarian	High M_r mucin	ID ₃	Gangopadhyay et al., Cancer Res., 45:1744, 1985
ovarian	High M_r mucin	DU-PAN-2	Lan et al, Cancer Res., 45:305, 1985
GY	7700 Kd GP	F 36/22	Croghan et al., Cancer Res., 44:1954, 1984
ovarian	'gp 68' 48 Kd GP	4F7/7A ₁₀	Bhattacharya et al., Cancer Res., 44:4528, 1984
GY	40, 42kD GP	OV-TL3	Poels et al., J. Natl. Cancer, 76:781, 1986
GY	'TAG-72' High M_r mucin	B72.3	Thor et al., Cancer Res., 46:3118, 1986

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
ovarian	300-400 Kd GP	DF ₃	Kufe et al., Hybridoma, 3:223, 1984
ovarian	60 Kd GP	2C ₈ /2F ₇	Bhattacharya et al., Hybridoma, 4:153, 1985
GY	105 Kd GP	MF 116	Mattes et al., PNAS, 81:568, 1984
ovarian	38-40 kD GP	MOv18	Miotti et al., Int. J. Cancer 39:297, 1987
GY	'CEA' 180 Kd GP	CEA 11-H5	Wagener et al., Int. J. Cancer, 33:469, 1984
ovarian	CA 19-9 or GICA	CA 19-9 (1116NS 19-9)	Atkinson et al., Cancer Res., 62:6820, 1982
ovarian	'PLAP' 67 Kd GP	H17-E2	McDicken et al., Br. J. Cancer, 52:59, 1985
ovarian	72 Kd	791T/36	Perkins et al., Eur. J. Nucl. Med., 10:296, 1985
ovarian	69 Kd PLAP	NDOG ₂	Sunderland et al., Cancer Res., 44:4496, 1984
ovarian	unknown M _r PLAP	H317	Johnson et al., Am. J. Reprod. Immunol., 1:246, 1981
ovarian	p185 ^{HER2}	4D5, 3H4, 7C2, 6E9, 2C4, 7F3, 2H11, 3E8, 5B8, 7D3, SB8	Shepard et al., J. Clin. Immunol., 11(3):117, 1991
uterus ovary	HMFG-2	HMFG2	Epenetos et al., Lancet, Nov. 6, 1000-1004, 1982
GY	HMFG-2	3.14.A3	Burchell et al., J. Immunol., 131:508, 1983
B: BREAST	330-450 Kd GP	DF3	Hayes et al., J. Clin. Invest., 75:1671, 1985
	NS	NCRC-11	Ellis et al., Histopathol., 8:501, 1984

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	37kD	3C6F9	Mandeville et al., Cancer Detect. Prev., 10:89, 1987
	NS	MBE6	Teramoto et al., Cancer, 50:241, 1982
	NS	CLNH5	Glassy et al., PNAS, 80:63227, 1983
	47 Kd GP	MAC 40/43	Kjeldsen et al, 2nd Int. Wkshop of MABs & Breast Cancer, San Fran., Nov. 1986
	High M _r GP	EMA	Sloane et al., Cancer, 17:1786, 1981
	High M _r GP	HMEG1 HFMG2	Arkie et al., Int. J. Cancer, 28:23, 1981
	NS	3.15.C3	Arkie et al., Int. J. Cancer, 28:23, 1981
	NS	M3, M8, M24	Foster et al., Virchows Arch. (Pathol. Anat. Histopathol.), 394:295, 1982
	1 (Ma) blood group Ags	M18	Foster et al., HumanPathol., 15:502, 1984
	NS	67-D-11	Rasmussen et al., Breast Cancer Res. Treat., 2:401, 1982
	oestrogen receptor	D547Sp, D75P3, H222	Kinsel et al., Cancer Res., 49:1052, 1989
	EGF Receptor	Anti-EGF	Sainsbury et al, Lancet, 1:364, 1985
	Laminin Receptor	LR-3	Horan Hand et al., Cancer Res., 45:2713, 1985
	<i>erb</i> B-2 p185	TA1	Gusterson et al., Br. J. Cancer, 58:453, 1988
	NS	H59	Hendler et al., Trans. Assoc. Am. Physicians, 94:217, 1981

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	126 Kd GP	10-3D-2	Soule et al., PNAS, 80:1332, 1983
	NS	HmAb1,2	Imam et al., cited in Schlom et al., Adv. Cancer Res., 43:143, 1985
	NS	MBR 1,2,3	Menard et al., Cancer Res., 63:1295, 1983
	95 Kd	24-17-1	Thompson et al., J. Natl. Cancer Inst., 70:409, 1983
	100 Kd	24-17-2 (3E1-2)	Croghan et al., Cancer Res., 43:4980, 1983
	NS	F36/22.M7/105	Croghan et al., Cancer Res., 44:1954, 1984
	24 Kd	Cl1, G3, H7	Adams et al., Cancer Res., 43:6297, 1983
	90 Kd GP	B6-2	Colcher et al., PNAS, 78:3199, 1981
	CEA & 180 Kd GP	B1-1	Colcher et al., Cancer Invest., 1:127, 1983
	colonic & pancreatic mucin similar to Ca 19-9	Cam 17-1	Imperial Cancer Research Technology MAb listing
	milk mucin core protein	SM3	Imperial Cancer Research Technology MAb listing
	milk mucin core protein	SM4	Imperial Cancer Research Technology MAb listing
	affinity-purified milk mucin	C-Mul (566)	Imperial Cancer Research Technology MAb listing

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	p185 ^{HER2}	4D5 3H4, 7C2, 6E9, 2C4, 7F3, 2H11, 3E8, 5B8, 7D3, 5B8	Shepard et al., J. Clin. Immunol., 11(3):117, 1991
	CA 125 > 200 Kd GP	OC 125	Kabawat et al., Int. J. Gynecol. Pathol., 4:245, 1985
	High M _r mucin/ glycolipid	MO v2	Miotti et al., Cancer Res., 45:826, 1985
	High M _r mucin	DU-PAN-2	Lan et al., Cancer Res., 44:1954, 1984
	'gp48' 48 Kd GP	4F7/7A ₁₀	Bhattacharya et al., Cancer Res., 44:4528, 1984
	300-400 Kd GP	DF ₃	Kufe et al., Hybridoma, 3:223, 1984
	'TAG-72' high M _r mucin	B72-3	Thor et al., Cancer Res., 46:3118, 1986
	'CEA' 180 Kd GP	ccccCEA 11	Wagener et al., Int. J. Cancer, 33:469, 1984
	'PLAP' 67 Kd GP	H17-E2	McDicken et al., Br. J. Cancer, 52:59, 1985
	HMEG-2 > 400 Kd GP	3-14-A3	Burchell et al., J. Immunol., 131:508, 1983
	NS	FO23C5	Riva et al., Int. J. Cancer, 2:114, 1988 (Suppl.)
<u>C: COLORECTAL</u>	TAG-72 High M _r mucin	B72-3	Colcher et al., Cancer Res., 47:1185 & 4218, 1987
	GP37	(17-1A) 1083-17-1A	Paul et al., Hybridoma, 5:171, 1986

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	Surface GP	CO17-1A	LoBuglio et al., JNCI, 80:932, 1988
	CEA	ZCE-025	Patt et al., Cancer Bull., 40:218, 1988
	CEA	AB2	Griffin et al., Proc. 2nd Conf. on Radioimmunodetection & Therapy of Cancer, 82, 1988
	cell surface AG	HT-29-15	Cohn et al., Arch. Surg. 122:1425, 1987
	secretory epithelium	250-30.6	Leydem et al., Cancer, 57:1135, 1986
	surface glycoprotein	44X14	Gallagher et al., J. Surg. Res., 40:159, 1986
	NS	A7	Takahashi et al., Cancer, 61:881, 1988
	NS	GA73-3	Munz et al., J. Nucl. Med., 27:1739, 1986
	NS	791T/36	Farrans et al., Lancet, 2:397, 1982
	cell membrane & cytoplasmic Ag	28A32	Smith et al., Proc. Am. Soc. Clin. O. col., 6:250, 1987
	CEA & vindesine	28.19.8	Corvalen, Cancer Immuno., 24:133, 1987
	gp72	X MMCO-791	Byers et al., 2nd Int. Conf. Mab Immunocon. Cancer, 41:1987
	high M_r mucin	DU-PAN-2	Lan et al., Cancer Res., 45:305, 1985
	high M_r mucin	ID ₃	Gangopadhyay et al., Cancer Res., 45:1744, 1985
	CEA 180 Kd GP	CEA 11-H5	Wagner et al., Int. J. Cancer, 33:469, 1984

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	60 Kd GP	2C ₈ /2F ₇	Bhattacharya et al., Hybridoma, 4:153, 1985
	CA-19-9 (or GICA)	CA-19-9 (1116NS 19-9)	Atkinson et al., Cancer Res., 62:6820, 1982
	Lewis a	PR5C5	Imperial Cancer Research Technology Mab Listing
	Lewis a	PR4D2	Imperial Cancer Research Technology Mab Listing
	colonic mucus	PR4D1	Imperial Cancer Research Technology Mab Listing
<u>D: MELANOMA</u>	p97 ^a	4-1	Woodbury et al., PNAS, 77:2183, 1980
	p97 ^a	8-2 M ₁₇	Brown, et al., PNAS, 78:539, 1981
	p97 ^b	96-5	Brown, et al., PNAS, 78:539, 1981
	p97 ^c	118-1, 133-2, (113-2)	Brown, et al., PNAS, 78:539, 1981
	p97 ^c	L ₁ , L ₁₀ , R ₁₀ (R ₁₉)	Brown et al., J. Immunol., 127:539, 1981
	p97 ^d	I ₁₂	Brown et al., J. Immunol., 127:539, 1981
	p97 ^e	K ₅	Brown et al., J. Immunol., 127:539, 1981
	p155	6-1	Loop et al., Int. J. Cancer, 27:775, 1981
	Gp3 disialogan- glioside	R24	Dippold et al., PNAS, 77:6115, 1980
	p210, p60, p250	5-1	Loop et al., Int. J. Cancer, 27:775, 1981
	p280 p440	225.28S	Wilson et al., Int. J. Cancer, 28:293, 1981
	GP 94, 75, 70 & 25	465.12S	Wilson et al., Int. J. Cancer, 28:293, 1981

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	P240-P250, P450	9-2-27	Reisfeld et al., Melanoma Ags & Abs, 1982 pp. 317 -
	100, 77, 75 Kd	F11	Chee et al., Cancer Res., 42:3142, 1982
	94 Kd	376.96S	Imai et al., JNCI, 68:761, 1982
	4 GP chains	465.12S	Imai et al., JNCI, 68:761, 1982; Wilson et al., Int. J. Cancer, 28:293, 1981
	GP 74	15-75	Johnson & Reithmuller, Hybridoma, 1:381, 1982
	GP 49	15-95	Johnson & Reithmuller, Hybridoma, 1:381, 1982
	230 Kd	Mel-14	Carrel et al., Hybridoma, 1:387, 1982
	92 Kd	Mel-12	Carrel et al., Hybridoma, 1:387, 1982
	70 Kd	Me3-TB7	Carrel et al., Hybridoma, 1:387, 1982
	HMW MAA similar to 9-2-27 AG	225.28SD	Kantor et al., Hybridoma, 1:473, 1982
	HMW MAA similar to 9-2-27 AG	763.24TS	Kantor et al., Hybridoma, 1:473, 1982
	GP95 similar to 376-96S 465-12S	705F6	Stuhlmiller et al., Hybridoma, 1:447, 1982
	GP125	436910	Saxton et al., Hybridoma, 1:433, 1982
	CD41	M148	Imperial Cancer Research Technology Mab listing

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
E: GASTROINTESTINAL			
pancreas, stomach	high M_r mucin	ID3	Gangopadhyay et al., Cancer Res., 45:1744, 1985
gall bladder, pancreas, stomach	high M_r mucin	DU-PAN-2	Lan et al., Cancer Res., 45:305, 1985
pancreas	NS	OV-TL3	Poels et al., J. Natl. Cancer Res., 44:4528, 1984
pancreas, stomach, oesophagus	'TAG-72' high M_r mucin	B72-3	Thor et al., Cancer Res., 46:3118, 1986
stomach	'CEA' 180 Kd GP	CEA 11-H5	Wagener et al., Int. J. Cancer, 33:469, 1984
pancreas	HMFG-2 > 400 Kd GP	3-14-A3	Burchell et al., J. Immunol., 131:508, 1983
G-I	NS	C COLI	Lemkin et al., Proc. Am. Soc. Clin. Oncol., 3:47, 1984
pancreas, stomach	CA 19-9 (or GICA)	CA-19-9 (1116NS 19-9) and CA50	Szymendera, Tumour Biology, 7:333, 1986
pancreas	CA125 GP	OC125	Szymendera, Tumour Biology, 7:333, 1986
E: LUNG			
non-small cell lung carcinoma	p185 ^{HER2}	4D5 3H4, 7C2, 6E9, 2C4, 7F3, 2H11, 3E8, 5B8, 7D3, SB8	Shepard et al., J. Clin. Immunol., 11(3):117, 1991

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
	high M_r mucin/ glycolipid	MO v2	Miolti et al., Cancer Res., 65:826, 1985
	'TAG-72' high M_r mucin	B72-3	Thor et al., Cancer Res., 46:3118, 1986
	high M_r mucin	DU-PAN-2	Lan et al., Cancer Res., 45:305, 1985
	'CEA' 180 kD GP	CEA 11-H5	Wagener et al, Int. J. Cancer., 33:469, 1984
Malignant Gliomas	cytoplasmic antigen from 85HG-22 cells	MUC 8-22	Stavrou, Neurosurg. Rev., 13:7, 1990
	cell surface Ag from 85HG-63 cells	MUC 2-63	Stavrou, Neurosurg. Rev., 13:7, 1990
	cell surface Ag from 86HG-39 cells	MUC 2-39	Stavrou, Neurosurg. Rev., 13:7, 1990
	cell surface Ag from 86HG-39 cells	MUC 7-39	Stavrou, Neurosurg. Rev., 13:7, 1990
G: MISCELLANEOUS	p53	PAb 240 PAb 246 PAb 1801	Imperial Cancer Research Technology MaB Listing
small round cell tumours	neural cell adhesion molecule	ERJC-1	Imperial Cancer Research Technology MaB Listing
medulloblastoma neuroblastoma rhabdomyosarcoma		M148	Imperial Cancer Research Technology MaB Listing

Table II - continued

Tumor Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
neuroblastoma		FMH25	Imperial Cancer Research Technology MaB Listing
renal cancer & glioblastomas	p155	6-1	Loop et al., Int. J. Cancer, 27:775, 1981
bladder & laryngeal cancers	"Ca Antigen" 350- 390 kD	CA1	Ashall et al., Lancet, July 3, 1, 1982
neuroblastoma	GD2	3F8	Cheung et al., Proc. AACR, 27:318, 1986
Prostate	gp48 48 kD GP	4F7/TA ₁₀	Bhattacharya et al., Cancer Res. 44:4528, 1984
Prostate	60 kD GP	2Cg/2F ₇	Bhattacharya et al., Hybridoma, 4:153, 1985
Thyroid	"CEA" 180 kD GP	CEA 11-H5	Wagner et al., Int. J. Cancer, 33:469, 1984

abbreviations: Abs, antibodies; Ags, antigens; EGF, epidermal growth factor; GI, gastrointestinal; GICA, gastrointestinal-associated antigen; GP, glycoprotein; GY, gynecological; HMFG, human milk fat globule; Kd, kilodaltons; Mabs, monoclonal antibodies; M_r, molecular weight; NS, not specified; PLAP, placental alkaline phosphatase; TAG, tumour-associated glycoprotein; CEA, carcinoembryonic antigen.

footnotes: the CA 19-9 Ag (GICA) is sialosylfucosyltetratetraosylceramide, also termed sialylated Lewis pentaglycosyl ceramide or sialylated lacto-N-fucopentaose II; p97 Ags are believed to be chondroitin sulphate proteoglycan; antigens reactive with Mab 9-2-27 are believed to be sialylated glycoproteins associated with chondroitin sulphate proteoglycan; unless specified, GY can include cancers of the cervix, endocervix, endometrium, fallopian tube, ovary, vagina or mixed Mullerian tumour; unless specified GI can include cancers of the liver, small intestine, spleen, pancreas, stomach and oesophagus.

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Generally speaking, antibodies of the present invention will preferably exhibit properties of high affinity, such as exhibiting a K_d of <200 nM, and preferably, of <100 nM, and will not show significant reactivity with life-sustaining normal tissues, such as one or more tissues selected from heart, kidney, brain, liver, bone marrow, colon, breast, prostate, thyroid, gall bladder, lung, adrenals, muscle, nerve fibers, pancreas, skin, or other life-sustaining organ or tissue in the human body. The "life-sustaining" tissues that are the most important for the purposes of the present invention, from the standpoint of low reactivity, include heart, kidney, central and peripheral nervous system tissues and liver. The term "significant reactivity", as used herein, refers to an antibody or antibody fragment, which, when applied to the particular tissue under conditions suitable for immunohistochemistry, will elicit either no staining or negligible staining with only a few positive cells scattered among a field of mostly negative cells. Many of the antibodies listed in Table II will not be of sufficient tumor specificity to be of use therapeutically. An example is MUC8-22 which recognizes a cytoplasmic antigen. Antibodies such as these will be of use only in diagnostic or investigational embodiments such as in model systems or screening assays.

Particularly promising antibodies from the standpoint of therapeutic application of the present invention are those having high selectivity for the solid tumor, such as B72.3, PR5C5 or PR4D2 for colorectal tumors; HMFG-2, TAG 72, SM-3, or anti-p 185^{Her2} for breast tumors; anti-p 185^{Her2} for lung tumors; 9.2.27 for melanomas; and MO v18 and OV-TL3 for ovarian tumors.

The listing of potential solid tumor cell surface antigen targets in Table II is intended to be

-55-

illustrative rather than exhaustive. Of course, in the practice of the invention, one will prefer to ensure in advance that the clinically-targeted tumor expresses the antigen ultimately selected. This is a fairly

5 straightforward assay, involving antigenically testing a tumor tissue sample, for example, a surgical biopsy, or perhaps testing for circulating shed antigen. This can readily be carried out in an immunological screening assay such as an ELISA, wherein the binding affinity of

10 antibodies from a "bank" of hybridomas are tested for reactivity against the tumor. Antibodies demonstrating appropriate tumor selectivity and affinity are then selected for the preparation of bispecific antibodies of the present invention. Antitumor antibodies will also be

15 useful in the preparation of antitumor antibody conjugates for use in combination regimens, wherein tumor endothelium and the solid tumor itself are both targeted (see, e.g., Figure 12).

20 E. PREPARATION OF IMMUNOTOXINS

While the preparation of immunotoxins is, in general, well known in the art (see, e.g., U.S. patents 4,340,535, and EP 44167, both incorporated herein by

25 reference), the inventors are aware that certain advantages may be achieved through the application of certain preferred technology, both in the preparation of the immunotoxins and in their purification for subsequent clinical administration. For example, while IgG based

30 immunotoxins will typically exhibit better binding capability and slower blood clearance than their Fab' counterparts, Fab' fragment-based immunotoxins will generally exhibit better tissue penetrating capability as compared to IgG based immunotoxins.

35

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Additionally, while numerous types of disulfide-bond containing linkers are known which can successfully be employed to conjugate the toxin moiety with the binding agent, certain linkers will generally be preferred over other linkers, based on differing pharmacologic characteristics and capabilities. For example, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at the site of action. Furthermore, while certain advantages in accordance with the invention will be realized through the use of any of a number of toxin moieties, the inventors have found that the use of ricin A chain, and even more preferably deglycosylated A chain, will provide particular benefits.

A wide variety of cytotoxic agents are known that may be conjugated to anti-endothelial cell antibodies. Examples include numerous useful plant-, fungus- or even bacteria-derived toxins, which, by way of example, include various A chain toxins, particularly ricin A chain, ribosome inactivating proteins such as saporin or gelonin, α -sarcin, aspergillin, restirictocin, ribonucleases such as placental ribonuclease, angiogenin, diphtheria toxin, and *pseudomonas* exotoxin, to name just a few. The most preferred toxin moiety for use in connection with the invention is toxin A chain which has been treated to modify or remove carbohydrate residues, so called deglycosylated A chain. The inventors have had the best success through the use of deglycosylated ricin A chain (dgA) which is now available commercially from Inland Laboratories, Austin, Tx.

However, it may be desirable from a pharmacologic standpoint to employ the smallest molecule possible that nevertheless provides an appropriate biological response.

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One may thus desire to employ smaller A chain peptides which will provide an adequate anti-cellular response. To this end, it has been discovered by others that ricin A chain may be "truncated" by the removal of 30
5 N-terminal amino acids by Nagarase (Sigma), and still retain an adequate toxin activity. It is proposed that where desired, this truncated A chain may be employed in conjugates in accordance with the invention.

10 Alternatively, one may find that the application of recombinant DNA technology to the toxin A chain moiety will provide additional significant benefits in accordance the invention. In that the cloning and
15 expression of biologically active ricin A chain has now been enabled through the publications of others (O'Hare et al., 1987; Lamb et al., 1985; Halling et al., 1985), it is now possible to identify and prepare smaller or
20 otherwise variant peptides which nevertheless exhibit an appropriate toxin activity. Moreover, the fact that ricin A chain has now been cloned allows the application of site-directed mutagenesis, through which one can
readily prepare and screen for A chain derived peptides and obtain additional useful moieties for use in
connection with the present invention.

25 The cross-linking of the toxin A chain region of the conjugate with the binding agent region is an important aspect of the invention. Where one desires a conjugate having biological activity, it is believed that a cross-
30 linker which presents a disulfide function is required. The reason for this is unclear, but is likely due to a need for the toxin moiety to be readily releasable from the binding agent once the agent has "delivered" the
toxin to the targeted cells. Each type of cross-linker,
35 as well as h w the cross-linking is performed, will tend to vary the pharmacodynamics of the resultant conjugate.

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Ultimately, one desires to have a conjugate that will remain intact under conditions found everywhere in the body except the intended site of action, at which point it is desirable that the conjugate have good "release" characteristics. Therefore, the particular cross-linking scheme, including in particular the particular cross-linking reagent used and the structures that are cross-linked, will be of some significance.

Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different proteins (e.g., a toxin and a binding agent). To link two different proteins in a step-wise manner, heterobifunctional cross-linkers can be used which eliminate the unwanted homopolymer formation. An exemplary heterobifunctional cross-linker contains two reactive groups: one reacting with primary amine group (e.g., N-hydroxy succinimide) and the other reacting with a thiol group (e.g., pyridyl disulfide, maleimides, halogens, etc.). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (e.g., the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (e.g., dgA).

The spacer arm between these two reactive groups of any cross-linkers may have various length and chemical composition. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (e.g., benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (e.g., disulfide bond resistant to reducing agents).

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The most preferred cross-linking reagent is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that steric hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to its delivery to the site of action by the binding agent. The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (e.g., the epsilon amino group of lysine). Another possible type of cross-linker includes the heterobifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

Although the "hindered" cross-linkers will generally be preferred in the practice of the invention, non-hindered linkers can be employed and advantages in accordance herewith nevertheless realized. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2-iminothiolane (Wawrzynczak & Thorpe, 1987). The use of such cross-linkers is well understood in the art.

Once conjugated, it will be important to purify the conjugate so as to remove contaminants such as unconjugated A chain or binding agent. It is important to remove unconjugated A chain because of the possibility of increased toxicity. Moreover, it is important to

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remove unconjugated binding agent to avoid the possibility of competition for the antigen between conjugated and unconjugated species. In any event, a number of purification techniques are disclosed in the

5 Examples below which have been found to provide conjugates to a sufficient degree of purity to render them clinically useful. In general, the most preferred technique will incorporate the use of Blue-Sepharose with a gel filtration or gel permeation step. Blue-Sepharose

10 is a column matrix composed of Cibacron Blue 3GA and agarose, which has been found to be useful in the purification of immunoconjugates (Knowles et al., 1987). The use of Blue-Sepharose combines the properties of ion exchange with A chain binding to provide good separation

15 of conjugated from unconjugated binding.

The Blue-Sepharose allows the elimination of the free (non conjugated) binding agent (e.g., the antibody or fragment) from the conjugate preparation. To

20 eliminate the free (unconjugated) toxin (e.g., dGA) a molecular exclusion chromatography step is preferred using either conventional gel filtration procedure or high performance liquid chromatography.

25 After a sufficiently purified conjugate has been prepared, one will desire to prepare it into a pharmaceutical composition that may be administered parenterally. This is done by using for the last purification step a medium with a suitable pharmaceutical

30 composition.

Suitable pharmaceutical compositions in accordance with the invention will generally comprise from about 10 to about 100 mg of the desired conjugate admixed with an

35 acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a final concentration

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of about 0.25 to about 2.5 mg/ml with respect to the conjugate. Such formulations will typically include buffers such as phosphate buffered saline (PBS), or additional additives such as pharmaceutical excipients, stabilizing agents such as BSA or HSA, or salts such as sodium chloride. For parenteral administration it is generally desirable to further render such compositions pharmaceutically acceptable by insuring their sterility, non-immunogenicity and non-pyrogenicity. Such techniques are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

A preferred parenteral formulation of the immunotoxins in accordance with the present invention is 0.25 to 2.5 mg conjugate/ml in 0.15M NaCl aqueous solution at pH 7.5 to 9.0. The preparations may be stored frozen at -10°C to -70°C for at least 1 year.

25

F. ATTACHMENT OF OTHER AGENTS

It is contemplated that most therapeutic applications of the present invention will involve the targeting of a toxin moiety to the tumor endothelium. This is due to the much greater ability of most toxins to deliver a cell killing effect as compared to other potential agents. However, there may be circumstances such as when the target antigen does not internalize by a route consistent with efficient intoxication by immunotoxins, where one will desire to target

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chemotherapeutic agents such as antitumor drugs, other cytokines, antimetabolites, alkylating agents, hormones, and the like. The advantages of these agents over their non-antibody conjugated counterparts is the added selectivity afforded by the antibody. One might mention by way of example agents such as steroids, cytosine arabinoside, methotrexate, aminopterin, anthracyclines, mitomycin C, vinca alkaloids, demecolcine, etoposide, mithramycin, and the like. This list is, of course, merely exemplary in that the technology for attaching pharmaceutical agents to antibodies for specific delivery to tissues is well established (see, e.g., Ghose & Blair, 1987).

It is proposed that particular benefits may be achieved through the application of the invention to tumor imaging. Imaging of the tumor vasculature is believed to provide a major advantage when compared to present imaging techniques, in that the cells are readily accessible. Moreover, the technology for attaching paramagnetic, radioactive and even fluorogenic ions to antibodies is well established. Many of these methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such as DTPA attached to the antibody (see, e.g., U.S. Patent 4,472,509). In the context of the present invention the selected ion is thus targeted to the tumor endothelium by the antibody, allowing imaging to proceed by means of the attached ion.

A variety of chemotherapeutic and other pharmacologic agents have now been successfully conjugated to antibodies and shown to function pharmacologically (see, e.g., Vaickus et al., 1991). Exemplary antineoplastic agents that have been investigated include doxorubicin, daunomycin, methotrexate, vinblastine, and various others (Dillman et

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al., 1988; Pietersz et al., 1988). Moreover, the attachment of other agents such as neocarzinostatin (Kimura et al., 1983), macromycin (Manabe et al., 1984), trenimon (Ghose, 1982) and α -amanitin (Davis & Preston, 1981) has been described.

In addition to chemotherapeutic agents, the inventors contemplate that the invention will be applicable to the specific delivery of a wide variety of other agents to tumor vasculature. For example, under certain circumstances, one may desire to deliver a coagulant such as Russell's Viper Venom, activated Factor IX, activated Factor X or thrombin to the tumor vasculature. This will result in coagulation of the tumor's blood supply. One can also envisage targeting a cell surface lytic agent such as phospholipase C (Flickinger & Trost, 1976) or cobra venom factor (CVF) (Vogel et al., 1981) which should lyse the tumor endothelial cells directly. The attachment of such structures to antibodies may be readily accomplished by protein-protein coupling agents such as SMPT. Moreover, one may desire to target growth factors, other cytokines or even bacterial endotoxin or the lipid A moiety of bacterial endotoxin to a selected cell type, in order, e.g., to achieve modulation of cytokine release. The attachment of such substances is again well within the skill in the art as exemplified by Ghose & Blair (1987).

Thus, it is generally believed to be possible to conjugate to antibodies any pharmacologic agent that has a primary or secondary amine group, hydrazide or hydrazine group, carboxyl alcohol, phosphate, or alkylating group available for binding or cross-linking to the amino acids or carbohydrate groups of the antibody. In the case of protein structures, this is most readily achieved by means of a cross linking agent (see preceding

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secti n on immunot xins). In the case of doxorubicin and daunomycin, attachment may be achieved by means of an acid labile acyl hydrazone or cis aconityl linkage between the drug and the antibody. Finally, in the case of methotrexate or aminopterin, attachment is achieved through a peptide spacer such as L-Leu-L-Ala-L-Leu-L-Ala, between the γ -carboxyl group of the drug and an amino acid of the antibody. For a general overview of linking technology, one may wish to refer to Ghose & Blair (1987).

The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

EXAMPLE I

A Murine Model for Antibody-Directed Targeting of Vascular Endothelial Cells in Solid Tumors

This example describes the development of a model system in which to investigate the antibody-directed targeting of vascular endothelial cells in solid tumors in mice. A neuroblastoma transfected with the mouse interferon- γ (IFN- γ) gene, C1300(Mu γ), was grown in SCID and antibiotic-treated BALB/c nude mice. The INF- γ secreted by the tumor induces the expression of MHC Class II antigens on the tumor vascular endothelium. Class II

MEM, modified Eagles medium; MHC, Major Hist compatibility Complex; SCID; severe combined immunodeficiency.

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antigens are absent from the vasculature of normal tissues, although they are present on B-lymphocytes, cells of monocyte/macrophage lineage and some epithelial cells. Intravenously-administered anti-Class II antibody strongly stains the tumor vasculature whereas an anti-tumor antibody, directed against a MHC Class I antigen of the tumor allograft, produces classical perivascular tumor cell staining.

10 A. MATERIALS AND METHODS

1. Animals

BALB/c *nu/nu* mice were purchased from Simonsen (Gilroy, CA). SCID mice were from the UT Southwestern Medical Center breeding colony. All animals were maintained in microisolation units on sterilized food and water. Where indicated, tetracycline - HCl (Vedco, St. Joseph, MO) was added to drinking water to a final concentration of 1.1 mg/ml (Harkness et al., 1983). Both strains carry the H-2^d haplotype.

2. Cells and Culture Conditions

All cell lines used in this study were cultured in modified Eagle's medium (MEM) supplemented with 10% (v/v) fetal calf serum, 2.4mM L-glutamine, 200 units/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10 CO₂. The C1300 neuroblastoma line was established from a spontaneous tumor which arose in an A/Jax mouse in 1940 (Dunham et al., 1953). The C1300(Muγ)12 line, hereafter abbreviated to C1300 (Muγ), was derived by transfection of C1300 cells with murine IFN-γ gene using the IFN-γ expression retrovirus pSVX (MuγδA₂) (Watanabe et al., 1988; Watanabe et al., 1989), and was cultured in

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MEM as above containing 1 mg/ml G418 (Geneticin, Sigma). Both lines carry the MHC haplotype H-2K^k, I-A^k, I-E^k, D^d. C1300 and C1300(Muγ) cells were grown in regular tissue culture flasks or, when large quantities were required for *in vivo* experiments, in cell factories (Baxter, Grand Prairie, TX). Cells from subcutaneous tumors were recovered for *in vitro* analysis by gentle mincing in MEM. After tumor cells had adhered overnight the monolayers were washed twice with MEM to remove nonadherent contaminant host cells.

Tumor conditioned media were prepared by seeding C1300 and C1300(MuAγ) cells at 25% of confluent density and culturing them for four days. Conditioned media were dialyzed for 16 hours against MEM without FCS to remove G418, filtered through a 0.22 μM membrane and stored at 4°C for no more than one week before assay. Aliquots of anti-IFN-γ antibodies (see 'Monoclonal Antibodies') sufficient to neutralize 200 international units (I.U.) of murine IFN-γ/ml of conditioned medium were added to some samples 24 hours before assay. The SVEC-10 murine endothelial cell line, hereafter abbreviated to SVEC, was kindly provided to Dr. M. Edidin, Department of Biology, Johns Hopkins University, Baltimore, MD and was derived by immortalization of lymph node endothelial cells from a C3H (H-2^k) mouse with SV40 (O'Connell et al., 1990). For some experiments, SVEC cells were cultured for 72 hours with 100 I.U./ml recombinant murine IFN-γ, (r-IFN-γ, a generous gift from Dr. F. Balkwill, Imperial Cancer Research Fund, London, England) or tumor-conditioned medium. In addition, 200 I.U./ml anti-IFN-γ antibody was added to some flasks at the beginning of the 72 hour culture period.

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3. Monoclonal Antibodies

The M5/114.15.2 (hereafter abbreviated to M5/114) and 11-4.1 hybridomas were purchased from the American Type Collection (Rockville, MD) and were grown in MEM-10% FCS. The antibodies were purified from culture supernatant by precipitation in 50% ammonium sulphate and affinity chromatography on Protein A. The rat IgG2b antibody, M5/114, detects an Ia specificity on I-A^b, I-A^q, I-A^d, I-E^d and I-E^k molecules (Bhattacharya et al., 1981). Thus, the antibody recognizes I-E^k molecules on SVEC (H-2^k) cells and I-A^d and I-E^d, hereafter referred to collectively as Ia^d, on cells from BALB/C nu/nu or SCID mice (both H-2^d). The anti-Ia^d reactivity of M5/114 was confirmed in this study by FACS analyses with the Ia^d expressing B-lymphoma line, A20/25 (Kim, 1978). The mouse IgG2a antibody 11-4.1 recognizes H-2K^k but not H-2K^d molecules (Oi et al., 1978) and so binds to H-2K^k on C1300 and C1300(Mu γ) cells but is unreactive with MHC antigens from BALB/c nu/nu or SCID mice. Isotype-matched control antibodies of irrelevant specificity were CAMPATH-2 (rat IgG2b, anti-human CD7 (Bindon et al., 1988) and WT-1 (mouse IgG2a, anti-human CD7 (Tax et al., 1984). Purified preparations of CAMPATH-2 and WT-1 were generous gifts from Dr. G. Hale (Department of Pathology, Cambridge, England) and Dr. W. Tax (Sint Radboudzeikenhuis, Nijmegen, the Netherlands) respectively.

Rat anti-mouse endothelial cell antibody MECA-20 (Duijvestijn et al., 1987) was donated as a concentrated culture supernatant by Dr. A. Duijvestijn (University of Limburg, the Netherlands) and used at a dilution of 1/200 for indirect immunoperoxidase staining. Rat antibodies against mouse macrophages (M1) and mouse CD3 (KT 31.1) were generously provided by Dr. P. Beverley (Imperial

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Cancer Research Fund, London, England). Hamster anti-mouse IFN- γ antibody 1222-00 (Sanchez-Madrid, 1983), used for specific neutralization of IFN- γ in vitro, was purchased from Genzyme (Boston, MA). Anti-mouse IFN- γ antibodies, XMG1.2 and R46A2, used in IFN- γ ELISAs, were kindly provided by Dr. N. Street (U.T. Southwestern Medical Center, Dallas, TX). Purified 11-4.1, WT-1 and XMG1.2 antibodies were biotinylated by incubation with a 12.5 fold molar excess of N-hydroxysuccinimidobiotin amidocaproate (Sigma) for one hour at room temperature followed by dialysis against two changes of PBS.

4. ELISA for murine IFN- γ

Sandwich ELISAs for murine IFN- γ were carried out as described previously (Cherwinski et al., 1989). In brief, the wells of flexible PVC microtiter plates (Dynatech, Alexandria, VA) were coated with 50 μ l/well of a 2 μ g/ml solution of capture anti-IFN- γ antibody, R46A2, in PBS for 2 hours at room temperature. Non-specific protein binding sites were blocked with 20% FCS in PBS for 15 minutes at 37°C. The plates were washed three times in PBS containing 0.05% (v/v) Tween 20 (Sigma) (PBS-T) and 25 μ l/well control and experimental samples in MEM-10% FCS were added. After incubating for 1 hour at 37°C, the wells were washed as before and 50 μ l/well of a 1 μ g/ml solution of biotinylated anti-IFN- γ antibody XMG1.2 in PBS-T containing 1% BSA were added. After incubation for 30 minutes at 37°C the wells were washed as before and incubated with 75 μ l of a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (DAKO) for one hour at room temperature. After thorough washing in PBS-T the wells were incubated for 30 minutes with 100 μ l/well of a 1 mg/ml solution of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) in citrate/phosphate buffer containing 0.003% (v/v) H₂O₂.

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Reaction product was measured as Abs.405 nm - Abs.490 nm. IFN- γ levels in experimental samples were calculated by reference to a recombinant murine IFN- γ standard solution in MEM-10% FCS.

5

5. Indirect Immunofluorescence

SVEC, C1300 and C1300(Mu γ) cells were prepared for FACS analyses as described previously (Burrows et al., 1991). All manipulations were carried out at room temperature. In brief, 50 μ l of a cell suspension at $2-3 \times 10^6$ cells/ml in PBS containing 0.2% (w/v) BSA and 0.2% (w/v) NaN₃ (PBS-BSA-N₃) were added to the wells of round-bottomed 96-well microtiter plates (Falcon 3910). Optimal dilutions of rat or mouse antibodies were distributed in 50 μ l volumes, and the plates sealed. After 15 minutes, the cells were washed four times by centrifuging the plates at 800 x g for 30 seconds, removing the supernatants, and resuspending the cells in 150 μ l/well PBS-BSA-N₃. Fluorescein isothiocyanate-conjugated rabbit antibodies against rat or mouse IgG (ICN, High Wycombe, England), diluted 1:20 in PBS-BSA-N₃ were distributed in 50 μ l volumes into the appropriate wells. The cells were incubated for a further 15 minutes and washed as before. Cell-associated fluorescence was measured on a FACScan (Becton Dickinson, Fullerton, CA). Data were analyzed using the CONSORT 30 program.

30

6. Preparation of tissues and immunohistochemistry

35

For the establishment of solid tumors, a total of 2×10^7 C1300 or C1300(Mu γ) cells, or a mixture of the two, in 200 μ l MEM-30% FCS were injected subcutaneously into the right anterior flank of BALB/c nu/nu or SCID mice. Tumor diameters were measured at regular intervals and the animals were euthanized after 16 days (rapidly-

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gr wing tumors) or 20 days (slowly-growing tumors). Tumors and normal tissues were excised immediately and snap-frozen over liquid nitrogen. Normal tissues were also harvested from non-tumor-bearing animals. Antibody localization experiments were performed in animals bearing 1 cm subcutaneous tumors induced by injection of C1300 and C1300(Muγ) in the ratio 7:3. One hundred micrograms of unconjugated M5/114 or CAMPATH-2 antibodies or 100 μg biotinylated 11-4.1 or WT-1 antibodies in 100 μl PBS were injected intravenously. At various times thereafter the animals were euthanized and their circulation was flushed with PBS for 5 minutes before removal and freezing of tumors and normal tissues as before. 8μM frozen sections were cut on a Tissuetek 2 cryostat (Baxter) and air-dried for 2 hours at room temperature. Slides were stored at -20°C for up to 3 months before assay.

Indirect immunoperoxidase staining for rat IgG was adapted from a method described previously (Billington et al., 1986). Briefly, sections were allowed to return to room temperature, air dried for 30 minutes and fixed in acetone for 15 minutes. After rehydration in PBS for 5 minutes, sections were incubated in a humidified chamber for 45-60 minutes with primary antibodies, diluted optimally in PBS-0.2% BSA, (PBS-BSA). After two washes in PBS, the sections were incubated for 30-45 minutes with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Carpinteria, CA) diluted 1:10 in PBS-BSA supplemented with 20% normal mouse serum (ICN, High Wycombe, UK) to block antibodies cross-reacting with mouse immunoglobulins. After a further two washes in PBS, the reaction product was developed using 0.5 mg/ml 3',3' - diaminobenzidine (Sigma) containing 0.01% (v/v) hydrogen peroxide for 8 minutes. The sections were counterstained with Mayer's hematoxylin (Sigma) for

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15 sec nds, dehydrated in absolute ethanol, cleared in xylene and mounted with Accumount 60 medium (Baxter). Indirect immunoperoxidase staining with biotinylated mouse antibodies was carried out in the same manner, except that peroxidase-conjugated streptavidin-biotin complex, diluted 1:50 in PBS with no blocking serum, was used as the second layer.

B. RESULTS

10

1. Murine IFN- γ levels in C1300(Mu γ) conditioned-medium

C1300(Mu γ)-conditioned medium contained 50.2 - 63.5 I.U./ml murine IFN- γ , in accordance with previous reports (Watanabe et al., 1989). By contrast, less than 5 I.U./ml IFN- γ was detected in C1300-conditioned medium or C1300(Mu γ)-conditioned medium to which an excess of neutralizing anti-IFN- γ antibody had been added 24 hours before assay.

20

2. Induction of MHC Class II (I-E^k) on SVEC cells by r.IFN- γ in C1300(Mu γ)-conditioned-medium

As shown in Fig. 1a, unstimulated SVEC cells did not express I-E^k. By contrast, a large majority of cells preincubated with r.IFN- γ (Fig. 1a) or with C1300(Mu γ)-conditioned medium (Fig. 1b) expressed significant levels of I-E^k, and this induction was almost completely blocked by anti-IFN- γ . Treatment of SVEC cells with r.IFN- γ or C1300(Mu γ)-conditioned medium did not cause non-specific antibody binding since the isotype-matched control antibody did not bind to the cells. These results were confirmed by indirect immunoperoxidase staining of cytospin preparations (n t shown).

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These findings suggested that vascular endothelial cells in tumors containing sufficient quantities of IFN- γ -secreting C1300(Mu γ) cells should be induced to express high cell surface levels of MHC Class II molecules.

5

3. Expression of MHC Class I (H-2K^k) and Class II (I-E^k) by C1300 and C1300(Mu γ) cells

Since IFN- γ can induce MHC Class II antigen expression in diverse cell types (Capobianchi et al. 1985; Collins et al., 1984; Hokland et al., 1988) and since the M5/114 antibody crossreacts with I-E^k, we determined whether the M5/114 antibody - intended for use to target tumor endothelial cells in vivo - would also bind to the tumor cells themselves. As shown in Fig. 2a, C1300(Mu γ) cells expressed I-E^k, but at levels 10-20 fold lower than those on SVEC cells stimulated with IFN- γ .

Similarly, C1300 cells expressed detectable but low levels of H-2K^k whereas C1300(Mu γ) cells displayed uniformly high levels, approximately 20-fold greater than on the parental line (Fig. 2b). This result was expected from the known autocrine Class I-inducing activity of IFN- γ and is in keeping with a previous report (Watanabe et al., 1989). Coculture of C1300(Mu γ) cells and C1300 cells induced homogeneous expression of I-E^k and H-2K^k on both populations (Fig. 2). Induction of these antigens on C1300 cells appears to be caused by IFN- γ released into the culture medium by the C1300(Mu γ) cells since the effect was centralized by anti-IFN- γ antibodies.

30

4. Growth of C1300 and C1300 (Mu γ) tumors in immunodeficient mice and induction of Ia^d on tumor vascular endothelial cells

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The inventors first attempted to grow subcutaneous C1300(Mu γ) tumors in BALB/c nu/nu and SCID mice because

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both strains carry the MHC haplotype (H-2^d) with which the anti-MHC Class II antibody M5/114 reacts, and because neither strain would be expected to reject the tumors, as do syngeneic immunocompetent A/J animals (Watanabe et al., 1989). For unknown reasons inocula composed entirely of C1300(Muγ) cells failed to produce progressively-growing tumors in BALB/c nu/nu or SCID mice. Conversely, pure C1300 inocula displayed 100% tumorigenicity but, as expected, did not contain Ia^d-positive endothelial cells.

In order to identify a combination which would yield a high percentage of tumor takes, reliable growth kinetics and cause Ia^d induction of a large majority of intratumoral endothelial cells, several ratios of C1300 and C1300(Muγ) cells were inoculated into BALB/c nu/nu mice. As shown in Fig. 3., mixtures containing C1300 and C1300(Muγ) cells in the ratio 9:1 produced rapidly-growing tumors but, when sections of the tumors were stained with anti-Ia^d antibody by the indirect immunoperoxidase technique, none of the endothelial cells in the tumor were found to be stained. Dropping the ratio of C1300: C1300(Muγ) to 8:2 gave rapidly-growing tumors in which approximately 50% of blood vessels were Ia^d-positive. Dropping the ratio further to 7:3 or 5:5 produced tumors which grew quite rapidly and contained a large majority of Ia^d-positive vessels. Dropping the ratio still further to 3:7 produced tumors in no more than half of the animals and those tumors that became palpable failed to grow beyond 6 mm in diameter. Histological analyses of the latter revealed no morphologically recognizable intact blood vessels and, hence, it was not possible to ascertain their level of Ia^d expression.

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Of the two usable C1300: C1300(Muy) ratios identified, 7:3 and 5:5, the ratio of 7:3 was adopted for the remainder of this study because the take rate was higher (100% vs. 80%) and the variability in tumor growth rate between individual animals was lower.

5. Distribution of Ia^d in BALB/c nude and SCID mice

The distribution of M5/114 binding in tissues from tumor-bearing BALB/c nu/nu mice is shown in Table III. In subcutaneous tumors, most or all vascular endothelial cells and numerous interstitial macrophages were stained. In most organs, the binding of M5/114 reflected the classical distribution of MHC Class II antigens, being restricted to B cells in lymphoid organs, resident macrophages in all tissues studied except brain and to tissue-specific elements of the reticuloendothelial system, such as liver Kupffer cells and Langerhans cells of the skin. In addition, staining was occasionally seen in some kidney tubules. When sections of small and large intestine from BALB/c nu/nu mice were examined, heavy labeling of both epithelial and endothelial cells was seen in both regions. By contrast, very little staining with M5/114 was seen in sections of intestine from SCID mice maintained in germ-free conditions. The staining of nu/nu mouse intestine was found to be related to the microbiological status of the animals and is discussed below. Apart from in the gut, no staining of endothelial cells with M5/114 was seen in any tissues examined in either nu/nu or SCID mice. The distribution of Ia^d antigens in normal tissues was not affected by the presence of the tumor because the staining pattern of M5/114 was identical in non-tumor-bearing mice.

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TABLE III.
Localization of Intravenously Administered anti-Ia^d Antibody
in C1300(Muγ) Tumor-Bearing Mice.^(a)

Tissue	Antigen Expression	Localization in vivo			
		1 hour	4 hours	24 hours	
Tumor ^(b)	Endothelial cells (EC), Mφ	EC ^(c)	EC	EC ^(d)	
Brain	None	None	None	None	
Colon ^(a)	Minority of epithelium & EC, Mφ	None	None	None	
Duodenum	Some epithelial cells & EC, Mφ	None	None	None	
Heart	Interstitial Mφ	None	None	None	
Kidney	Occasional proximal tubule, Mφ	None	None	None	
Liver	Kupffer cells (KC), numerous Mφ in parenchyma	KC ^(c)	KC	KC ^(d) some Mφ	
Lung	Numerous Mφ in parenchyma	None	None	None	
Pancreas	Numerous Mφ in parenchyma	None	None	None	
Skin ^(c)	Langerhans cells	None	None	None	
Spleen	Red pulp (RP) Mφ, marginal zone (MZ) B cells & Mφ, some T/B cells in PALS	MZ	MZ PALS	MZ, RP PALS	

(a) Experiments performed with SCID or antibiotic-treated BALB/c nu/nu mice.

(b) Mixed tumor of 7:3 C1300:C1300(Muγ) cells grown subcutaneously.

(c) Strong staining, including discernable labelling of luminal membranes.

(d) Weaker staining, entirely intracellular.

(e) Either adjacent to, or distant from tumor.

PALS: Periaarteriolar lymphatic sheath, Mφ: Macrophages

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6. Attenuation of expression of Ia^d on colonic endothelium and epithelium of nude mice by administration of antibiotics

5 In BALB/c nu/nu mice, most epithelial cells from all regions of the gut were intensely stained with anti-Ia^d antibody. In addition, some endothelial cells in both upper and lower bowel bound M5/114 antibody, particularly those associated with colonic villi. When the animals
10 were treated with oral tetracycline-Hcl, a broad-spectrum antibiotic, for 1-3 weeks there was a progressive diminution of Ia^d expression in the colon and elsewhere in the gut, so that binding of M5/114 was in most sections restricted to the luminal membranes of a
15 minority of epithelial cells. Light cytoplasmic staining of occasional endothelial cells was observed in some antibiotic-treated animals. The pattern of epithelial and endothelial Ia^d expression was not homogeneus and the intensity of M5/114 staining correlated with the
20 frequency of CD3+ T lymphocytes in the adjacent lamina propria. Antibiotic treatment was associated with a dramatic decrease in the numbers of intravillous CD3-positive cells: after three weeks practically all had disappeared from the underlying parenchyma and associated
25 lymphoid deposits and there was a coincident decline in Ia^d expression on surrounding epithelial and endothelial cells.

30 In SCID mice, epithelial and endothelial cell Ia^d expression and T-cell infiltration of the colon resembled that of antibiotic-treated BALB/c nu/nu animals.

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7. Specific localization of intravenously administered anti-Ia^d antibody to tumor vasculature, B cells and macrophages in SCID and antibiotic-treated nude mice

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Tumor-bearing BALB/c nu/nu and SCID mice were given intravenous injections of anti-Ia^d or the isotype-matched control antibody and euthanized 1, 4 or 24 hours later. The *in vivo* localization of anti-Ia^d antibody in tumor and normal tissues is shown in Table III. Anti-Ia^d antibody was found on the luminal membrane and in the cytoplasm of most or all tumor vascular endothelial cells one hour after injection. A similar pattern was seen at four hours after injection, but by 24 hours the labeling of tumor endothelial cells was weaker and entirely intracellular, consistent with the progressive internalization and metabolism of the antibody by endothelial cells (Table III). Also, at 24 hours small amounts of antibody were detectable in the immediate perivascular regions of the tumor.

Anti-Ia^d antibody was bound to Kupffer cells in the intravascular compartment of the liver within one hour of injection. At later times after injection, internalization and degradation of the antibody was apparent (Table III). Adjacent sinusoidal endothelial cells were not stained. The high permeability of hepatic fenestrated endothelia was indicated by the penetrance of the antibody to reach some hepatic parenchymal macrophages (Table III). In the spleen, perivascular B cells and macrophages in white pulp marginal zones were stained within one hour, showing that the vasculature of this organ was particularly permeable to antibody. At later stages the antibody penetrated throughout the splenic lymphoid compartment and also labelled a minority of red pulp macrophages (Table III). In organs other than the liver and spleen, macrophages and related cells

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such as the Langerhans cells of the skin were unstained probably because their vascular endothelium contains tight junctions and is relatively impermeable to antibodies.

5

Anti-Ia^d antibody was bound to some endothelial cells in the colon of BALB/c nu/nu mice, but not elsewhere in the intestine, one hour after injection. Antibiotic treatment for 1-3 weeks before injection of anti-Ia^d antibody completely abolished localization to gut endothelial cells. No intravenously injected anti-Ia^d antibody homed to gut endothelia in SCID mice. The isotype-matched control antibody was not detected in tumor or normal tissues at any time after injection.

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Taken together, these results strongly indicate that, when injected into appropriate tumor-bearing animals anti-Ia^d antibody or immunoconjugates will localize effectively to most or all tumor endothelial cells while sparing life-sustaining normal tissues.

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8. Perivascular staining of tumor cells in mice injected with anti-tumor (H-2K^k) antibody

When frozen sections of subcutaneous tumors deriving from inocula of mixed C1300 and C1300(Muγ) cells (7:3) were stained with biotinylated anti-H-2K^k antibody, a homogeneous staining pattern was obtained. The levels of IFN-γ secreted by the C1300(Muγ) cells in the tumor were therefore sufficient to induce increased H-2K^k expression by the C1300 component of the tumor, in accordance with the in vitro co-culture experiments described above. The staining was specific because no staining was seen with the isotype-matched control antibody. No specific labeling of any normal tissue by anti-H-2K^k antibody was

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found, as expected since this antibody was raised in an H-2^d mouse strain.

In contrast with the rapid binding of intravenously-administered anti-Ia^d antibody to tumor vasculature, no significant accumulation of anti-H-2K^k antibody was apparent one hour after injection. After four hours, however, anti-H-2K^k antibody was detected in small islands of tumor cells surrounding central capillaries. After 24 hours, the antibody was bound to larger discrete areas of tumor cells but staining intensity was diminished relative to the earlier time points. Each with localization times of up to 72 hours, homogenous labeling of all tumor cells was not achieved.

No localization of anti-H-2K^k antibody was found in any normal tissues and binding of the isotype-matched control antibody was not detectable in tumor or normal tissues.

20

C. DISCUSSION

This example describes a murine model for studying the antibody-directed targeting of vascular endothelial cells in solid tumors. In this model, IFN- γ gene-transfected tumor cells growing in SCID or antibiotic-treated nude mice release IFN- γ which induces the *de novo* expression of MHC Class II antigens on the tumor vasculature. MHC Class II is absent from the vasculature in the normal tissues of these mice and hence the Class II induced on the tumor vascular endothelial cells serves as a specific marker. Class II is present on B-lymphocytes, Kupffer cells and other cells of monocyte/macrophage lineage but these cells are not life-sustaining so their temporary absence after targeting with cytotoxic immunoconjugates should be tolerable.

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IFN- γ also induces the tumor cells themselves to express high levels of the MHC Class I antigen, H-2K^k, which can serve as a tumor cell-specific marker in BALB/c nu/nu or SCID mice, which both carry the H-2K^d haplotype. Thus, anti-Ia^d and anti-H-2K^k antibodies injected systemically localize selectively to tumor vascular endothelial cells and tumor cells respectively, which enables the approaches of targeting the tumor vasculature and the tumor cells to be compared in this model, or used in combination.

It was necessary to dilute the C1300(Mu γ) cells with C1300 parental cells in the ratio 3:7 to establish progressively-growing subcutaneous tumors in which the vascular endothelial cells were Class II (Ia^d) - positive. Undiluted C1300(Mu γ) cells were poorly tumorigenic in BALB/c nu/nu mice, in contrast with a prior report (Watanabe et al., 1989). Vascular dysfunction appeared to be the reason why pure C1300(Mu γ) tumors would not grow beyond a diameter of 5-6 mm. Staining of sections of tumors with the anti-endothelial cell antibody MECA 20 revealed that the vessels were morphologically atypical with no visible lumens. It is possible that excessively high intratumoral IFN- γ levels in pure C1300(Mu γ) tumors caused direct vascular toxicity or activated macrophages in the tumor to become cytotoxic for endothelial cells (Peri et al., 1990).

Intravenously injected anti-Ia^d antibody bound rapidly and homogeneously to vascular endothelial cells in the tumor, confirming the immediate accessibility of intravascular targets (Kennel et al., 1991). Remarkably, the inductive influence of IFN- γ from C1300(Mu γ) cells was completely restricted to the tumor mass: endothelial cells in the overlying skin expressed no detectable Ia^d and did not bind any intravenously-

-81-

injected anti-Ia^d antibody. It is likely that IFN- γ entering the systemic circulation is neutralized by a specific binding protein, perhaps a soluble form of the IFN- γ receptor (Novick et al., 1989), whose normal role may be to down-regulate cytokine activity (Fernandez-Botran et al., 1991) or to restrict it to the immediate locale of secretion.

Ia^d antigens are not restricted solely to tumor endothelial cells. MHC Class II antigens are expressed constitutively by B cells, activated T cells and cells of the monocyte/macrophage lineage in humans and rodents (Daar et al., 1984; Hämmerling et al., 1976) and were found in this study also to be present on occasional proximal tubules in the kidney and on some epithelial cells in the intestine of SCID and antibiotic-treated BALB/c nu/nu mice. However, when injected intravenously, only the hepatic Kupffer cells, splenic B cells and macrophages in the liver and spleen bound detectable amounts of the anti-Ia^d antibody: the potentially life-sustaining Class II-positive renal and gut epithelial cells were unstained. Localization of intravenously-injected anti-Ia^d antibody to hepatic Kupffer cells and splenic marginal zone B cells occurred within one hour, in accordance with the report of Kennel et al. (1991). Presumably, the extreme permeability of the discontinuous splenic endothelium permits rapid extravasation of antibodies into the parenchyma of this organ and staining of the marginal zone B-cells (Kennel et al., 1991).

The reason for the lack of staining of renal and gut epithelial cells is probably that these cells are not readily accessible to intravenously-administered antibody because the antibody would have to diffuse across basement membranes and several tissue layers to reach these cells. In addition, it is likely that all the

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remaining anti-Ia^d antibody in the circulation was absorbed by more accessible splenic white pulp lymphocytes before significant extravasation into the red pulp (Kennel et al., 1991; Fujimori et al., 1989) or other normal tissues could occur. This is important because it illustrates a potentially critical pharmacokinetic difference between vascular targeting and tumor cell targeting. Because the tumor endothelial cells are so accessible to intravenously-administered antibody, the presence of a large 'sink' of competing antigen in the blood or lymphoid organs should not prevent the antibody from reaching the target cells but should protect antigen-positive cells in most extravascular compartments. It is conceivable that an antibody recognizing a tumor vascular endothelial cell antigen that is shared by epithelial cells, for instance, might be targeted without the toxic side-effects which have complicated therapy with anti-tumor cell immunoconjugates (Spitler, 1988). Furthermore, even in the absence of such a sink, it is possible that operative specificity for tumor endothelial cells could be achieved in the face of cross-reactivity with extravascular normal tissues by decreasing the dose or by using rapidly-cleared antibody fragments in the construction of the immunoconjugate.

Although anti-Ia^d antibody did not localize to life-sustaining Ia^d+ extravascular tissues such as kidney tubules and gut epithelium, it did bind to colonic endothelial cells in non-antibiotic-treated BALB/c nu/nu mice. These cells were as accessible as tumor endothelial cells and were required for survival since regular BALB/c nu/nu mice treated with high doses of M5/114 immunotoxins died from intestinal damage. Murine endothelial cells do not express MHC Class II antigens in vitro (O'Connell et al., 1990; Duijvestijn et al., 1986)

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or *in vivo* (de Waal et al., 1983) unless stimulated with IFN- γ so it is likely that induction of Ia^d on intestinal endothelial and epithelial cells was a result of local secretion of IFN- γ by helper T cells (Cherwinski et al.
5 1987) or activated NK cells (Anegon, 1988; Kasahara, 1983) in response to gut flora. In accordance with this view, numerous CD3+, CD8+ T cells were observed in the villous stroma and their frequency correlated with the intensity of staining of endothelial and epithelial cells
10 with anti-Ia^d antibody. Furthermore, oral administration of tetracycline-Hcl (a broad spectrum antibiotic) reversed T cell infiltration, diminished Ia^d expression and abolished localization of intravenously-injected anti-Ia^d antibody to colonic endothelial cells.

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Antibiotic treatment had no effect on Ia^d expression by tumor endothelial cells. In subsequent experiments it was found that SCID mice had little Ia^d on colonic epithelial or endothelial cells and that intravenously-
20 administered anti-Ia^d antibody did not localize to their colonic endothelium. Furthermore, high doses of M5/114 immunotoxins were non-toxic in these animals. Given the possibility of antibiotic resistance arising in the gut flora of tetracycline-treated BALB/c *nu/nu* mice, we
25 believe that SCID mice may be more suitable for these types of experiments.

Consistent with the findings of others (Baxter et al., 1991; Kennel et al., 1991; Jones et al., 1988;
30 Pervez et al., 1988), an anti-tumor antibody directed against the H-2K^k antigen on C1300 and C1300(Mu γ) cells showed perivascular staining of tumor cells after intravenous administration. In view of the homogeneous expression of H-2K^k by tumor cells *in vitro* and in
35 sections of subcutaneous tumors, it is likely that the uneven intratumoral distribution of intravenously-

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injected anti-H-2K^k antibody was related to the vascular and interstitial physiology of the tumor (Jain, 1990; Fujimori et al., 1989). This nicely demonstrates, in a single system, the limitations of using antitumor antibodies for targeting and the virtues of tumor vascular targeting. It may be possible to combine both approaches to advantage because the tumor cells that survive destruction of intratumoral blood vessels are likely to be those at the periphery of the tumor mass, close to the tumor-host interface. These areas are likely to be well vascularized by capillaries in adjacent normal tissues and have low interstitial pressure (Jain, 1990), so the surviving cells should be amenable to attack by antitumor immunoconjugates.

In summary, the inventors describe a murine model with which to test the feasibility of targeting the vasculature of solid tumors. The model permits the antitumor effects of immunoconjugates directed against tumor vasculature to be compared with those of immunoconjugates directed against the tumor cells themselves.

EXAMPLE II

Solid Tumor Therapy Using A Vascular Targeted Immunotoxin

This example describes the successful therapy of the solid tumor model described in Example I, using the anti-tumor endothelial cell immunotoxin, MS/114dgA, and the anti-tumor cell immunotoxin, 11-4.1dgA, alone as well as in combination therapy against large solid tumors.

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A. MATERIALS AND METHODS**1. Animals**

5 BALB/c nu/nu mice were purchased from Simonsen
(Gilroy, CA). SCID mice were from the National Cancer
Institute (Bethesda, MD). Germ-free SCID mice were from
the University of Wisconsin (Madison, WI). All animals
were maintained in microisolation units on sterilized
10 food and water.

2. Cells and Culture Conditions

All cell lines used in this study were cultured in
15 modified Eagle's medium (MEM) supplemented with 10% (v/v)
fetal calf serum, 2.4mM L-glutamine, 200 units/ml
penicillin and 100 µg/ml streptomycin, 100 µM non-
essential amino acids, 1 µM Na Pyruvate and 18 µM HEPES.
Cultures were maintained at 37°C in a humidified
20 atmosphere of 90% air/10% CO₂. The C1300 neuroblastoma
line was established from a spontaneous tumor which arose
in an A/Jax mouse in 1940 (Dunham et al., 1953). The
C1300(Muγ)12 line, hereafter abbreviated to C1300 (Muγ),
was derived by transfection of C1300 cells with murine
25 IFN-γ gene using the IFN-γ expression retrovirus PSVX
(MuγΔA₅) (Watanabe et al., 1988), and was cultured in MEM
as above containing 1 mg/ml G418 (Geneticin, Sigma).
Both lines carry the MHC haplotype H-2K^k, I-A^k, I-E^k, D^d.
C1300 and C1300(Muγ) cells were grown in regular tissue
30 culture flasks or, when large quantities were required
for in vivo experiments, in cell factories (Baxter, Grand
Prairie, TX). Cells from subcutaneous tumors were
recovered for in vitro analysis by gentle mincing in MEM.
After tumor cells had adhered overnight the monolayers
35 were washed twice with MEM to remove nonadherent
contaminant host cells.

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Tumor conditioned media were prepared by seeding C1300 and C1300(MuA γ) cells at 25% of confluent density and culturing them for four days. Conditioned media were dialyzed for 16 hours against MEM without FCS to remove G418, filtered through a 0.22 μ M membrane and stored at 4°C for no more than one week before assay. Aliquots of anti-IFN- γ antibodies (see 'Monoclonal Antibodies') sufficient to neutralize 200 international units (I.U.) of murine IFN- γ /ml of conditioned medium were added to some samples 24 hours before assay. The SVEC-10 murine endothelial cell line, hereafter abbreviated to SVEC, was kindly provided to Dr. M. Edidin, Department of Biology, Johns Hopkins University, Baltimore, MD and was derived by immortalization of lymph node endothelial cells from a C3H (H-2^k) mouse with SV40 (O'Connell et al., 1990). For some experiments, SVEC cells were cultured for 72 hours with 100 I.U./ml recombinant murine IFN- γ , (r-IFN- γ , a generous gift from Dr. F. Balkwill, Imperial Cancer Research Fund, London, England) or tumor-conditioned medium. In addition, 200 I.U./ml anti-IFN- γ antibody was added to some flasks at the beginning of the 72 hour culture period.

3. Monoclonal Antibodies

The M5/114.15.2 (hereafter abbreviated to M5/114) and 11-4.1 hybridomas were purchased from the American Type Collection (Rockville, MD) and were grown in MEM-10% FCS. The antibodies were purified from culture supernatant by precipitation in 50% ammonium sulphate and affinity chromatography on Protein G (M5/114) or Protein A (11-4.1). The rat IgG2b antibody, M5/114, detects an Ia specificity on I-A^b, I-A^q, I-A^d, I-E^d and I-E^k molecules (Bhattacharya et al., 1981). Thus, the antibody recognizes I-E^k molecules on SVEC (H-2^k) cells and I-A^d and I-E^d, hereafter referred to collectively as

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Ia^d, on cells from BALB/C nu/nu or SCID mice (both H-2^d). The mouse IgG2a antibody 11-4.1 recognizes H-2K^k but not H-2K^d molecules (Oi et al., 1978) and so binds to H-2K^k on C1300 and C1300(Muγ) cells but is unreactive with MHC antigens from BALB/c nu/nu or SCID mice. Isotype-matched control antibodies of irrelevant specificity were CAMPATH-2 (rat IgG2b, anti-human CD7 (Bindon, 1988) and WT-1 (mouse IgG2a, anti-human CD7 (Tax et al., 1984). Purified preparations of CAMPATH-2 and WT-1 were obtained from Dr. G. Hale (Department of Pathology, Cambridge, England) and Dr. W. Tax (Sint Radboudzeikenhuis, Nijmegen, the Netherlands) respectively.

4. Preparation of dgA

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The ricin A chain was purified by the method of Fulton et al. (Fulton et al., 1986), deglycosylated ricin A was prepared as described by Thorpe et al., (1985). For conjugation with antibodies, the A chain was reduced with 5 mM DTT and subsequently separated from DTT by gel filtration on a column of Sephadex G-25 in PBS, pH 7.5.

5. Preparation of Immunotoxins

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IgG immunotoxins were prepared using the 4-succinimidylloxycarbonyl-α-methyl(1-pyridyldithio)toluene linking agent described by Thorpe et al. (1987). Briefly, 4-succinimidylloxycarbonyl-α-methyl(2-pyridyldithio)toluene dissolved in dimethylformamide was added to the antibody solution (7.5 mg/ml in borate buffer, pH 9.0) to give a final concentration of 0.11 mM. After 1 hour the derivatized protein was separated from unreacted material by gel chromatography on a Sephadex G-25 column and mixed with freshly reduced ricin A chain. The solution was concentration to about 3 mg/ml and

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allowed to react for 3 days. Residual thiol groups were inactivated by treating the immunotoxin with 0.2 mM cysteine for 6 hours. The solution was then filtered through a Sephacryl S-200 HR column in 0.1 M phosphate buffer, pH 7.5, to remove unreacted ricin A, cysteine, and aggregates. Finally, the immunotoxin was separated from free antibody by chromatography on a Blue Sepharose CL-6B column equilibrated in 0.1 M sodium phosphate buffer, pH 7.5, according to the method of Knowles and Thorpe (1987).

6. Cytotoxicity Assays

C1300, C1300(Mur) and SVEC cells suspended at 10^5 cells/ml in MEM-10% FCS were distributed in 100 μ l volumes into the wells of flat-bottomed microtiter plates. For some assays, SVEC cells were suspended in C1300- or C1300(Mur)-conditioned medium or MEM supplemented with 100 I.U./ml r.IFN- γ as indicated. Immunotoxins in the same medium were added (100 μ l/well) and the plates were incubated for 24 hours at 37°C in an atmosphere of 10% CO₂ in humidified air. After 24 hours, the cells were pulsed with 2.5 μ Ci/well [³H] leucine for another 24 hours. The cells were then harvested onto glass fiber filters using a Titertek harvester and the radioactivity on the filters was measured using a liquid scintillation spectrometer (LKB; Rackbeta). The percentage of reduction in [³H] leucine incorporation, as compared with untreated control cultures, was used as the assessment of killing.

7. Antitumor Studies

For the establishment of solid tumors, a mixture of 1.4×10^7 C1300 cells and 6×10^6 C1300(Mur) cells in 200 μ l MEM-30% FCS were injected subcutaneously into the

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right anterior flank of BALB/c nu/nu or SCID mice. Fourteen days later, when the tumors had grown to 0.8-1.2 cm in diameter, the mice were separated into groups of 5-10 animals and injected intravenously with 200 µl of immunotoxins, antibodies or diluent. Perpendicular tumor diameters were measured at regular intervals and tumor volumes were estimated according to the following equation (Steel, 1977).

10 volume - $\frac{\text{Smaller diameter}^2 \times \text{larger diameter} \times \pi}{6}$

For histopathological analyses, animals were euthanized at various times after treatment and the tumors were excised immediately into 4% (v/v) formalin. Paraffin sections were cut and stained with hematoxylin and eosin or Massons trichrome.

20 B. RESULTS

The first studies carried out involved a comparison of killing activity of anti-Class II immunotoxin (M5/114 dgA) against unstimulated SVEC mouse endothelial cells with those stimulated with conditioned medium from IFN-γ-secreting tumor cells (C1300 Muγ). These studies were carried out in order to demonstrate that the anti-Class II immunotoxin, M5/114 dgA exerts a selective toxicity against IFN-γ stimulated endothelial cells, and not against unstimulated cells. The results are shown in Figures 4a and b. In Figure 4a, SVEC mouse endothelial cells were cultured in regular medium and the cultured cells subjected to varying immunotoxin concentrations as indicated. As will be appreciated, while ricin effected a 50% inhibition of leucine incorporation at about 3×10^{-11} , neither the anti-Class II immunotoxin (M5/114 dgA) nor the control immunotoxin (CAMPATH-2 dgA) exerted a significant toxic effect within the concentration

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ranges tested. In contrast, when the SVEC mouse endothelial cells were stimulated by culturing in the presence of C1300(Mu γ)-conditioned medium, the mouse endothelial cells became quite sensitive to the anti-Class II immunotoxin, with 50% of stimulated cells being killed by the anti-Class II immunotoxin at a concentration of about 3×10^{-10} M. Thus, these studies demonstrate that γ interferon, which is produced by the C1300(Mu γ) and present in the conditioned media effectively promote the appearance of Class II targets on the surface of the SVEC cells.

Figure 5 illustrates similar studies, which confirm the finding that the C1300(Mu γ) conditioned media effectively promotes the expression of Class II molecules on endothelial cells. In particular, the data shown in Figure 5 demonstrate that both recombinant IFN- γ as well as conditioned media from C1300(Mu γ) sensitize endothelial cells to the anti-tumor endothelial cell immunotoxin, M5/114 dgA. Figure 5 also demonstrates that conditioned media from C1300 cells that do not secrete interferon (C1300 TCM), as well as interferon-producing C1300 cells (Mu γ) pretreated with anti-IFN- γ , both do not promote an anti-Class II immunotoxin sensitizing effect.

25

Next, a series of studies were carried out wherein the killing activity of an anti-Class I (anti-tumor) immunotoxin (11-4.1-dgA) and that of the anti-Class II immunotoxin (M5/114-dgA) are compared against a 70:30 mixed population of C1300 and C1300(Mu γ) cells. Figure 6a simply demonstrates that in a 70:30 culture of C1300 and C1300(Mu γ), that only the anti-Class I immunotoxin, 11-4.1-dgA, and ricin, exert a cytotoxic effect. Figure 6b shows killing of cells freshly recovered from subcutaneous tumors in mice. Taken together, these figures demonstrate that the anti-tumor immunotoxin kills

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tumor cells well, but that the anti-tumor endothelial cell immunotoxin does not. Thus, any anti-tumor effect of M5/114-dgA would not likely be due to direct tumor cell killing. Therefore, these studies serve as a control for later studies wherein it is demonstrated that M5/114-dgA can have a profound anti-tumor effect in the solid tumor model system described in Example I, through an anti vascular effect.

Figure 7 also shows a comparison of killing of pure and mixed populations of C1300 and C1300(Muγ) by the anti-tumor cell immunotoxin 11-4.1 dgA (anti-H-2K^k). Both figures show the effects of the anti-tumor cell immunotoxin against four different tumor populations. Again, in each case the anti-tumor cell immunotoxin demonstrate significant anti-tumor activity, at a concentration of on the order of about 10^{-10} M. Thus, these data show that mixed tumors should be highly sensitive to the anti-tumor immunotoxin, a control that is needed in order to demonstrate the anti-vascular attributes of the anti-Class II immunotoxin.

The next series of studies involved the application of one or both of the foregoing anti-Class I and anti-Class II immuno-toxins, in the model tumor system disclosed in Example I. Figure 8 illustrates the anti-tumor effects of the anti-tumor endothelial cell immunotoxin, M5-114 dgA. As can be seen, dosages as low as 20 μg exhibited a noticeable antitumor effect. While the change in mean tumor volume in the 20 μg-treated population does not, in Figure 8, appear to be particularly dramatic, sections of the tumor, when H & E-stained, illustrated surviving "islands" of tumor cells in a "sea" of necrotic cells. This can be seen in Figure 9, wherein the surviving islands of tumor cells are the

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darker staining areas, and the necrotic tissue the more lightly-staining areas.

Importantly, treatment with 40 μ g of M5/115-dgA resulted in dramatic anti-tumor effects, as can be seen in Figure 8, and marked regressions were achieved in all animals. Here, 30 days after tumor inoculation the mean tumor volume equated with the 16 day figure in the controls. The dotted line in Figure 8 represents the results that were expected with the use of 100 μ g of M5/114 dgA, with a possible reoccurrence of tumor cell indicated at the 26 day position being the partial result of a surviving rim of viable cells observed in the treated solid tumor. It was found that tumors collapsed to an average of a quarter of their initial volume, sometimes reaching almost unmeasurable dimensions, before regrowing 7 - 10 days later.

The gross appearance of tumors treated with M5/114-dgA changed dramatically within two days. Massive hemorrhaging of the tumor vascular bed caused the tumors to assume a blackened, bruised appearance reminiscent of the effects seen when Meth-A fibrosarcomas are treated with bacterial endotoxin (Carswell et al., 1975) or tumor necrosis factor- α (TNF α) (Havell et al., 1988). Over the next 5-7 days, the tumors collapsed to form a flat scabrous plug that subsequently detached, leaving a small avascular area of scar tissue in animals treated with M5/114-dgA + 11-4.1-dgA. Some tumors became fully coagulated before significant hemorrhaging could occur and so progressed to the final stage illustrated without blackening or scabbing of the tumor mass. In mice given M5/114-dgA alone, the tumors regrew from a ring around the original site and reinvaded the core of dead tumor tissue.

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A study of the time course of the events in tumor-bearing mice treated with M5/114-dgA revealed vascular endothelial cell destruction as the first visible event, occurring as early as 2 hours after administration of the immunotoxin. Degeneration of the endothelial cell layer induced a wave of platelet adhesion and activation followed by fibrin deposition. By 6 hours, many blood vessels in the tumor were occluded with thrombi and had been stripped of their endothelial cell lining. At this time, the tumor cells themselves were morphologically unchanged. By 24 hours, all vessels contained mature thrombi and the surrounding tumor cells had pyknotic nuclei, and by 48 hours, massive tumor cell degeneration and autolysis had occurred.

Figure 10 is a section through a 1.2 cm tumor 72 hours after treatment with 100 μ g of the anti-Class II immunotoxin M5/114 dgA, followed by H & E staining. As can be seen, this pattern is similar to the 20 μ g data shown in Figure 9, but certainly much more dramatic in that virtually no "islands" of tumor cells remain. It is estimated that this pattern represents a complete necrosis of greater than 95% of the tumor diameter, leaving only a thin cuff of surviving tumor cells, presumably nourished by vessels in overlying skin.

To address this potential source of recurrence, i.e., the potential for a cuff of surviving tumor cells, combined therapy with both an antitumor (anti-Class I) and an anti-endothelial (anti-Class II) immunotoxin was undertaken. The theory for this combined therapeutic approach can be seen in Figure 11, which illustrates the appearance of the solid tumor following 48-72 hours of intravenous immunotoxin treatment. At the left hand side of the figure is represented a tumor following anti-tumor immunotoxin therapy alone. As illustrated, only those

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areas immediately surrounding the blood vessels become necrotic following treatment with the anti-tumor immunotoxin, due to the inability of the immunotoxin to sufficiently infiltrate the tumor and reach the tumor cells that are distal of the blood vessels. In stark contrast, shown in the middle panel of Figure 11 is a representation of the low dose treatment with anti-endothelial cell immunotoxin. Here is illustrated the effects of a low dose of the anti-endothelial cell immunotoxin, which results in necrosis of the tumor in those parts distal of the blood vessels, except for the outer rim of the tumor which is presumably fed by associated normal tissues. At low dosages, only those areas of the tumor closest to the blood vessels will receive sufficient oxygen and nutrients. Next, the high dose anti-endothelial immunotoxin results are illustrated on the right hand side of Figure 11. Here, the only living tumor remaining is that associated with the outer rim of the tumor. It was a goal of combined therapy studies to demonstrate an additive or even synergistic effect when both an anti-tumor immunotoxin and anti-endothelial cell immunotoxin were employed in combination. This effect is illustrated in the panel at the right hand side of Figure 11.

25

The results of this combination therapy are shown in Figure 12. Figure 12 shows the anti-tumor effects of the anti-tumor immunotoxin (11-4.1-dgA) alone at a high dose, the anti-tumor endothelial cell immunotoxin (M5/114-dgA) alone at a low dose, as well as combinations of both. The results demonstrate that both immunotoxins had a transient but noticeable effect in and of themselves, with the anti-tumor immunotoxin showing a slightly greater anti-tumor effect than the anti-tumor endothelial cell immunotoxin, although this might be a dosing effect.

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Truly dramatic synergistic results were seen when both were used in combination. When 100 μ g of the anti-tumor immunotoxin was given on day 14, followed by 20 μ g of the anti-tumor endothelial cell immunotoxin on day 16, one out of four cures were observed. When the order of administration was reversed, i.e., the anti-tumor endothelial cell immunotoxin given first, even more dramatic results were observed, with two out of four cures realized. The latter approach is the more logical in that the initial anti-endothelial cell therapy serves to remove tumor mass by partial necrosis, allowing better penetration into the tumor of the anti-tumor immunotoxin.

Therapeutic doses (≥ 40 μ g) of M5/114-dgA did not cause detectable damage to Class II-positive epithelial cells or to hepatic Kupffer cells, as assessed by histopathological analysis at various times after treatment. Any lymphoid cells destroyed by M5/114-dgA were apparently replaced from bone marrow precursors because, 20 days after treatment, all mature bone marrow cell populations and splenic B cell compartments were normal.

C. DISCUSSION

The findings from this model validate the concept of tumor vascular targeting and, in addition, demonstrate that this strategy is complimentary to that of direct tumor targeting. The theoretical superiority of vascular targeting over the conventional approach was established by comparing the *in vivo* antitumor effects of two immunotoxins, one directed against tumor endothelium, the other against the tumor cells themselves, in the same model. The immunotoxins were equally potent against their respective target cells *in vitro* but, while 100 μ g of the tumor-specific immunotoxin had practically no effect against large solid C1300(Muy) tumors, as little

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as 40 µg of the anti-tumor endothelial cell immunotoxin caused complete occlusion of the tumor vasculature and dramatic tumor regressions.

5 Despite causing thrombosis of all blood vessels within the tumor mass, the anti-tumor endothelial cell immunotoxin was not curative because a small population of malignant cells at the tumor-host interface survived and proliferated to cause the observed relapses 7-10 days
10 after treatment. The proximity of these cells to intact capillaries in adjacent skin and muscle suggests that they derived nutrition from the extratumoral blood supply, but the florid vascularization and low interstitial pressure in those regions of the tumor
15 rendered the surviving cells vulnerable to killing by the anti-tumor immunotoxin (Jain, 1990; Weinstein & van Osdol, 1992), so that combination therapy produced some complete remissions.

20 The time course study demonstrated that the anti-Class II immunotoxin exerted its antitumor activity via the tumor vasculature since endothelial cell detachment and diffuse intravascular thrombosis clearly preceded any changes in tumor cell morphology. In contrast with the
25 anti-tumor immunotoxin, the onset of tumor regression in animals treated with the anti-tumor endothelial cell immunotoxin was rapid. Massive necrosis and tumor shrinkage were apparent in 48-72 hours after injection. Focal denudation of the endothelial lining was evident
30 within 2-3 hours, in keeping with the fast and efficient *in vivo* localization of M5/114 antibody and the endothelial cell intoxication kinetics of the immunotoxin ($t_{1/10} = 2$ hours, $t_{1/2} = 12.6$ hours).

35 As only limited endothelial damage is required to upset the hemostatic balance and initiate irreversible

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coagulation, many intratumoral vessels were quickly thrombosed with the result that tumor necrosis began within 6-8 hours of administration of the immunotoxin. This illustrates several of the strengths of vascular targeting in that an avalanche of tumor cell death swiftly follows destruction of a minority of tumor vascular endothelial cells. Thus, in contrast to conventional tumor cell targeting, anti-endothelial immunotoxins could be effective even if they have short serum half lives and only bind to a subset of tumor endothelial cells.

MHC Class II antigens are also expressed by B-lymphocytes, some bone marrow cells, myeloid cells and some renal and gut epithelia in BALB/c *nu/nu* mice, however, therapeutic doses of anti-Class II immunotoxin did not cause any permanent damage to these cell populations. Splenic B cells and bone marrow myelocytes bound intravenously injected anti-Class II antibody but early bone marrow progenitors do not express Class II antigens and mature bone marrow subsets and splenic B cell compartments were normal 3 weeks after therapy, so it is likely that any Ia⁺ myelocytes and B cells killed by the immunotoxin were replaced from the stem cell pool. It is contemplated that the existence of large numbers of readily accessible B cells in the spleen prevented the anti-Class II immunotoxin from reaching the relatively inaccessible Ia⁺ epithelial cells but hepatic Kupffer cells were not apparently damaged by M5/114-dgA despite binding the immunotoxin. Myeloid cells are resistant to ricin A-chain immunotoxins, probably due to unique endocytic pathways related to their degradative physiologic function (Engert et al., 1991).

No severe vascular-mediated toxicity was seen in the studies reported here because mice were maintained on

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oral antibiotics which minimized immune activity in the small intestine.

The findings described in this example demonstrate the therapeutic potential of the vascular targeting strategy against large solid tumors. As animal models for cancer treatment are widely accepted in the scientific community for their predictive value in regard to clinical treatment, the invention is also intended for use in man. Numerous differences between tumor blood vessels and normal ones have been documented (Denekamp, 1990; Engert et al., 1991; Jain, 1988) and are envisioned to be of use in practicing this invention. Tumor endothelial markers may be induced directly by tumor-derived angiogenic factors or cytokines (Ruco et al., 1990; Burrows et al., 1991) or could relate to the rapid proliferation (Denekamp & Hobson, 1982) and migration (Folkman, 1985a) of endothelial cells during neovascularization. Candidate anti-tumor endothelial cell antibodies include FB-5, against endosialin (Rettig et al., 1992), and E9 (Kumar et al., 1993) which are reportedly highly selective for tumor vascular endothelial cells. Two related antibodies developed by the present inventors, TEC-4 and TEC-11, against carcinoma-stimulated human endothelial cells, show strong reactivity against vascular endothelial cells in a wide range of malignant tumors but little or no staining of vessels in benign tumors or normal tissues. Vascular targeting is therefore envisioned to be a valuable new approach to the therapy of disseminated solid cancers for which there are currently no effective treatments.

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Example III**Targeting the Vasculature of Murine Breast Tumors**

5

This example describes a proposed approach for targeting the vasculature of breast cancer and other solid tumors in humans. This approach is exemplified through the use of bispecific antibodies to selectively induce the activation antigens, Class II and ELAM-1, on the vascular endothelial cells of syngeneic breast tumors in mice and then targeting these antigens with immunotoxins. The murine models employed will parallel the situation in humans. If successful vascular targeting is achieved in the mouse, it should also be possible in man since highly specific anti-breast cancer antibodies are available (Denekamp, 1984; Girling et al., 1989; Griffin et al., 1989; Lan et al., 1987; Boyer et al., 1989). In the case of clinical (as opposed to diagnostic application), the central issue is to confine the expression of the induced target antigen to tumor vasculature.

In the case of Class II, which is present on the vasculature of normal tissues in mice and humans (Natali et al., 1981; Daar et al., 1984; Hammerling, 1976), the objective is to suppress its expression throughout the vasculature and then selectively induce it on tumor vasculature. In the case of ELAM-1, which is absent from the vasculature of normal tissues (Cotran et al., 1976), the objective is to induce its expression selectively on tumor vasculature.

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A. Overview**1. Selective Induction of Class II Expression on Tumor Vasculature**

5

C3H/He mice will be injected subcutaneously with syngeneic MM102 mammary tumor cells. The tumor cells express Ly6.2 which is a unique marker in C3H mice (Ly6.1 positive). Mice bearing solid MM102 mammary tumors will be treated with CsA to reduce or abolish Class II expression throughout the vasculature. As originally shown in the dog (Groenewegen et al., 1985), and, as recently confirmed by the inventors in the mouse, CsA inhibits T cell and NK cell activation and lowers the basal levels of IFN- γ to the extent that Class II disappears from the vasculature. The mice will then be injected with a bispecific (Fab'-Fab') anti-CD28/anti-Ly6A.2 antibody, which should localize to the tumor by virtue of its Ly6.2-binding activity. The bispecific antibody should then bind to T cells which are present in (or which subsequently infiltrate (Blanchard et al., 1988) the tumor. Crosslinking of CD28 antigens on the T cells by multiple molecules of bispecific antibody attached to the tumor cells should activate the T cells via the CsA-resistant CD28 pathway (Hess et al., 1991; June et al., 1987; Bjorndahl et al., 1989). Activation of T cells should not occur elsewhere because the crosslinking of CD28 antigens which is necessary for activation (Thompson et al., 1989; Koulova et al., 1991) should not occur with soluble, non-tumor cell bound, bispecific antibody. T cells which become activated in the tumor should release IFN- γ which should induce Class II antigens on the tumor vascular endothelium (Collins et al., 1984; Pober et al., 1983) and probably on the tumor cells themselves (Boyer et al., 1989). Animals will then be treated with anti-Class II immunotoxins to destroy the tumor blood supply.

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2. Induction of ELAM-1 Expression on Tumor Vasculature

Mice bearing solid MM102 mammary tumors will be injected with bispecific (Fab'-Fab') anti-CD14/anti-Ly6A.2 antibody. The antibody should localize in the tumor by virtue of its Ly6.2-binding activity. It should then activate monocytes and macrophages in the tumor by crosslinking their CD14 antigens (Schutt et al., 1988; Chen et al., 1990). The activated monocytes/macrophages should have tumoricidal activity (Palleroni et al., 1991) and release IL-1 and TNF which should rapidly induce ELAM-1 antigens on the tumor vascular endothelial cells (Bevilacqua et al., 1987; Pober et al., 1991). A monoclonal antibody to mouse ELAM-1 will be generated and used as an immunotoxin to destroy the tumor blood supply.

B. EXPERIMENTAL DESIGN AND METHODS

20 1. Suppression of Class II Expression

a) Mouse mammary tumors, MM102 and MM48.

The tumors preferred for use are the mouse mammary (MM) tumors which have been extensively characterized by Dr. Reiko Irie and colleagues (Irie, 1971; Irie et al., 1970). The inventors have obtained from Dr. Irie (UCLA School of Medicine, CA) two transplantable tumors, MM102 and MM48. The MM102 line derives from a spontaneous mammary tumor which originated in a C3H/He mouse. The MM102 tumor carries an antigen which is closely related to, or identical to, Ly6A.2 (Seto, et al., 1982). Since the C3H/He mouse expresses Ly6.1 and not Ly6.2, this marker is tumor-specific in syngeneic mice. The MM48 tumor, a variant of the original tumor, lacks Ly6A.2 and provides a specificity control in the proposed studies.

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Both tumors form continuously-growing solid tumors when injected into the subcutaneous site.

b) Monoclonal antibodies.

5

For targeting the Ly6A.2 antigen, the inventors have obtained the anti-Ly6A.2 hybridoma, S8.106, from Dr. Ulrich Hammerling (Memorial Sloan-Kettering Cancer Center, N.Y.). This hybridoma secretes a mouse IgG_{2a} antibody (Kimura, et al., 1980) which has been shown to react specifically with MM102 and other Ly6A.2 expressing MM tumors (Seto, et al., 1982).

15 The anti-mouse CD28 antibody (Gross, et al., 1990) we shall use was raised by Dr. James Allison (University of California, CA), who also has provided ascitic fluid from hybridoma-bearing animals for synthesizing the bispecific antibody. The antibody is a hamster IgG.

20 Isotype-matched negative control antibodies will be the WT1 antibody (anti-human CD7) which is a mouse IgG_{2a} and a hamster IgG of irrelevant specificity from the ATCC.

25 Antibodies will be purified on staphylococcal Protein A coupled to Sepharose, or by ion exchange and size exclusion chromatography on Sepharose 4B as described previously (Ghetie, et al., 1988). The ability of the purified anti-Ly6A.2 antibody to bind to MM102 cells and of the anti-CD28 antibody to bind mouse T cells will be confirmed by FACS analyses as described previously (Burrows, et al., 1991).

35 Purified antibodies will be filtered through 0.22 μ membranes, aliquotted, and stored at -70°C.

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c) Preparation of Fab' fragments:

F(ab')₂ fragments of purified anti-Ly6A.2 and anti-CD28 antibodies will be prepared by pepsin digestion, as described by Glennie et al. (1987). Purified antibodies (5-10 mg) will be dialyzed against 0.1 M sodium acetate, pH 4.1, and digested with 4% (w/w) pepsin at 37°C. The digestion will be followed by SDS-PAGE and terminated by raising the pH when optimal digestion is achieved. Undigested IgG will be removed by chromatography on a Sephacryl S-200 column equilibrated with PBS. The F(ab')₂ fragments will be analyzed by SDS-PAGE and, if detectable levels of undigested antibody should remain, the F(ab')₂ fragments will be further purified by removal of undigested antibody on a Protein A-Sepharose column. Fab' fragments will be prepared from F(ab')₂ fragments by reduction with 5 mM DTT for 1 hr at 25°C, followed by removal of free DTT on a Sephadex G-25 column equilibrated against phosphate-EDTA buffer (Glennie et al., 1987)

d) Preparation of anti-Ly6A.2/anti-CD28 bispecific antibodies:

For the production of anti-Ly6A.2-anti-CD28 bispecific antibodies, Fab' fragments of each antibody will be initially prepared as above and will be left unalkylated. Heterodimer molecules will be prepared as described by Glennie et al. (1987). Briefly, Fab' fragments will be reduced with DTT in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA for 60 min. at room temperature. One of the Fab' fragments will be then reacted with Ellman's reagent (2mM) for 1 hour at room temperature in acetate buffer, pH 5.0. The free Ellman's reagent will be separated using a Sephadex G-25 column. The derivatized Fab' fragment will be then mixed with the

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other reduced Fab' and allowed to react at room temperature for 24 hours. Bispecific antibodies will be separated from remaining Fab' fragments by gel filtration over Sephacryl S-200 columns.

5

e) **Confirmation of cell binding-capacity of anti-Ly6A.2/anti-CD28 bispecific-antibody:**

FACS analyses will be performed to verify the dual
10 cell-binding capacity of the bispecific antibody. MM102 tumor cells (grown as an ascites) will be treated for 30 minutes at 4°C with the bispecific antibody ($10 \mu\text{g}/10^6$ cells) and washed. The tumor cells will then be incubated with fluorescenated goat anti-hamster
15 immunoglobulin for 30 minutes at 4°C and washed again. The fluorescence associated with the cells will then be measured using the FACS. Positive staining of tumor cells coated with bispecific antibody and lack of staining of cells coated with anti-Ly6A.2 antibody alone
20 will confirm that the bispecific antibody is intact and is capable of binding tumor cells. The experiment will be repeated using a CD28 positive mouse T cell lymphoma line (e.g., EL4) and with fluoresceinated goat anti-mouse immunoglobulin as the detecting antibody to confirm that
25 the bispecific antibody has CD28-binding capacity.

f) **Activation of T cells by anti-Ly6A.2/anti-CD28 bispecific antibody plus MM102 tumor cells:**

30

It will be important to confirm that tumor cells coated with the bispecific antibody, but not free bispecific antibody, are able to activate T cells in a CsA-resistant fashion. T cells will be enriched from the
35 spleens of C3H/He mice by depleting B-cells and macrophages according to the procedure of Lee et al., 1990 (Lee, et al., 1990). Spleen cells are treated with

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mouse anti-Class II antibody and the Class II-expressing cells are removed by treating them with goat anti-mouse IgG-coupled magnetic beads and withdrawing them with a strong magnet. The non-adherent cells are decanted and are treated further to remove residual B cells and macrophages by successive rounds of treatment with anti-J11D plus BRC and anti-MAC-1 antibody plus goat anti-rat serum. After these procedures, the remaining cells are $\geq 95\%$ T cells and $< 3\%$ Ig positive.

10

T cells will be cultured (0.5 to 1×10^5 cells/ 0.2 ml) in medium in the wells of 96-well plates. Various concentrations of anti-CD28 IgG, anti-CD28 Fab' or anti-Ly6A.2/anti-CD28 bispecific antibody will be added together with various concentrations of one of the following costimulants: PMA, IL1 or anti-CD3 IgG. CSA ($0.5 \mu\text{g/ml}$) will be added to an identical set of cultures. The cultures will be incubated at 37°C for 3 days, ^3H -thymidine ($1 \mu\text{Ci/culture}$) will be added and the plates harvested 24 hours later. These experiments should confirm that bivalent anti-CD28, but not monovalent Fab' anti-CD28 or the bispecific antibody, stimulate T cells and that the stimulation is not CSA inhibitable.

25

Next, MM102 and MM48 cells, obtained from ascitic tumors of C3H/He mice, will be treated with mitomycin C ($25 \mu\text{g/ml}$) for 20 minutes at 37°C . The cells will then be washed and the above experiment repeated with the inclusion of 0.5 to 1×10^5 mitomycin-treated MM102 or MM48 cells along with the T cells in the cultures. The MM102 cells, but not the MM48 cells, should present the bispecific antibody to the T cells and, together with the costimulant, induce their stimulation.

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- g) Confirmation that injection of anti-Ly6A.2/anti-CD28 bispecific antibody into CsA-treated MM102 tumor-bearing mice results in induction of Class II selectively on tumor vasculature:

5

C3H/He mice will be injected subcutaneously with 10^6 MM102 or MM48 tumor cells. One day later they will start daily treatments with CsA (60 mg/kg/day) given either orally dissolved in olive oil or injected intraperitoneally. After 10-14 days, when the tumors will have reached 1.0-1.3 cm in diameter, and when Class II will have disappeared from the vasculature, mice will be injected with 50-100 μ g of anti-Ly6A.2/anti-CD28 bispecific antibody. Other mice will receive various control treatments, including unconjugated anti-Ly6A.2 or anti-CD28 (Fab' and IgG) or diluent alone. Two or three days later, the mice will be sacrificed and the tumors and various normal tissues will be removed for immunohistochemical examination. Frozen sections will be cut and stained for the presence of Class II antigens and for the presence of hamster immunoglobulin using indirect immunoperoxidase techniques, as presented in the foregoing examples.

25

Upon demonstration that Class II antigens are strongly and selectively expressed on the vasculature of MM102 tumors but not on MM48 tumors, the tumor therapy studies below will be carried out. If Class II antigens are absent from tumor vasculature but hamster immunoglobulin is present, this would indicate that the bispecific antibody had localized to the tumor, as anticipated from prior studies with analogous bispecific antibodies (Perez, et al., 1985; Garrido, et al., 1990), but that T cell activation had not occurred sufficiently for IFN- γ secretion to ensue. If so, the presence of T cells will be verified by staining frozen tumor sections with anti-CD28 and anti-CD3 antibodies. If T cells are

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present, again as would be anticipated from prior studies (Koulova, et al., 1991; Perez, et al., 1985), the failure to get Class II induction might be attributable to the need for two signals for T cell activation, i.e. a 2nd
5 signal might be missing. This will be checked by coadministering an anti-Ly6A.2/anti-CD3 bispecific antibody, which together with the anti-Ly6A.2/anti-CD28 bispecific, should provide the signalling needed for T cell activation.

10

h) Synthesis of anti-class II-SMPT-dgA immunotoxin:

Immunotoxins directed against murine class II MHC
15 molecules will be prepared by linking the rat monoclonal anti-murine I-A^k antibody to deglycosylated ricin A (dgA) using the previously described disulfide cleavable crosslinker, SMPT (Thorpe, et al., 1988). Briefly, affinity purified antibody molecules will be derivatized
20 by reaction with a five-fold molar excess of SMPT in borate buffer, pH 9.0, for 60 min. at room temperature. Free SMPT will be removed by passage through a Sephadex G-25 column equilibrated against phosphate buffered saline containing EDTA (1mM, PBSE). Under these
25 conditions, an average of 1.7 molecules of SMPT are introduced per immunoglobulin molecule. Next, the derivatized antibody will be allowed to react with reduced dgA for 72 hrs. at room temperature. Under these conditions, immunoconjugates form through formation of
30 disulfide linkage between sulfhydryl groups on dgA molecules and SMPT. Immunoconjugates will be separated from free dgA by gel filtration in Sephacryl S-200 columns and from unreacted antibody by passage through Blue-Sepharose and elution with phosphate buffer
35 containing 0.5M NaCl. Purity of immunoconjugates will be assessed by SDS-PAGE.

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i) Tumor therapy experiments.

C3H/He mice will be injected subcutaneously with 10^6 MM102 or MM48 tumor cells and, one day later, will start daily treatments with CsA (60 mg/kg/d). When the tumors have grown to 1.0-1.3 cm diameter, the mice will receive an intravenous injection of 50-100 μ g anti-Ly6A.2/anti-CD28 bispecific antibody (perhaps together with anti-Ly6A.2/anti-CD3 bispecific antibody if indicated by the experiments in Section (h) above). Two or three days later, 100 μ g of the anti-Class II immunotoxin will be administered intravenously. Anti-tumor effects will be monitored by measuring the size of the tumors at regular intervals and by histological examination as in Section C. The specificity of any anti-tumor effects will be established by comparing the anti-tumor effects with those in mice which receive various control treatments, including unconjugated anti-Ly6A.2 Fab' and IgG, unconjugated anti-CD28 Fab' and IgG, and anti-Class II immunotoxin alone.

2. Induction of ELAM-1 on tumor vasculature by anti-Ly6A.2/anti-CD14 bispecific antibody.**a) Raising of anti-ELAM-1 monoclonal antibodies.****i) Induction of ELAM-1 on SVEC cells for immunization:**

30

Expression of cytokine-induced adhesion molecules on SVEC murine endothelial cells will be induced by stimulation of SVEC cell monolayers with a cocktail of rMuIL- 1β (50 I.U./ml), rMuTNF α (100 IU/ml) and bacterial endotoxin (100 ng/ml) for 4 hrs at 37°C, as previously described for the induction of human ELAM-1 (Bevilacqua, et al., 1987). Preliminary evidence suggests that SVEC

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cells activated in this manner express murine ELAM-1, since radiolabeled U937 cells, which bear the ELAM-1 agent (D. Simmons, personal communication), display increased adhesion to activated SVEC cells within 2 hrs of the addition of the cytokines. The increased endothelial cell adhesiveness peaked at 4-6 hrs., as previously reported for human ELAM-1. Although cytokine-activated SVEC cells also displayed long-term (up to 48 hrs.) adhesiveness to U937 cells, this was probably not due to ICAM-1/LFA-1 or VCAM-1/VLA-4 interactions, since the assays were carried out at 4°C, under shear stress conditions, which inhibits any adhesive interactions other than those between selections and their carbohydrate agents (Spertini, et al., 1991). Increased adhesiveness at the later time points was probably mediated by the selection LAM-1 (Mel-14) on U937 cells and its agent (the MECA 79 antigen) on SVEC cells (81). In subsequent experiments, this pathway will be blocked by the inclusion of Mel-14 and/or MECA 79-specific antibodies in the adhesion assays (Imai, et al., 1991).

ii) Immunization:

Rat monoclonal antibodies will be raised against inducible proteins on mouse endothelial cells. SVEC cells will be stimulated for 6 hrs., as previously described, before immunization of Wistar rats. The rats will be boosted three weeks following the initial injection with identically-prepared SVEC cells. Serum from the injected rats will be tested for the presence of antibodies specific for induced proteins on endothelial cells 7-10 days after the second boost, using FACS analysis of induced and non-induced SVEC cells. Additional boosting and screening will be repeated as necessary.

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Once antibody levels have been detected in acceptable titers, rats will be given a final boost with induced SVEC cells and their spleens removed after 3 days. Splenocytes will be fused with Y3 Ag1.2.3 rat myeloma cells according to standard protocols using poly-ethylene glycol 4000 (82). Hybridomas will be selected using HAT medium and the supernatants screened using FACS analysis.

10 iii) Screening:

Those hybridomas secreting antibodies reacting with cytokine-induced but not with resting SVEC cells will be selected for further characterization. Reactivity will be assessed by indirect immunofluorescence with hybridoma supernatants and FITC-labeled mouse anti-rat immunoglobulin antibodies. The selected hybridomas will be expanded, and the immunoglobulin purified from their culture supernatants. Confirmation of ELAM-1 reactivity will be carried out as described below.

iv) Characterization of antigens:

The physicochemical properties of the precipitated antigens will be investigated after activating SVEC cells with cytokines in the presence of cycloheximide or tunicamycin and by immunoprecipitation of antibody-reactive molecules from lysates of ³⁵S-methionine-labeled SVEC cells, using the selected MoAbs. Immunoprecipitates will be subsequently analyzed by SDS-PAGE. Confirmation of murine ELAM-1 reactivity will be carried out by comparison of the precipitated material and human ELAM-1 using SDS-PAGE, one-dimensional proteolytic maps with staphylococcal V8 protease and NH₂-terminal sequences.

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**b) Preparation of anti-Ly6A.2-anti-CD14
bispecific antibodies**

Bispecific antibodies will be constructed using Fab' fragments derived from anti-Ly6A.2 and anti-CD14 monoclonal antibodies, essentially as described in the previous section. Several anti-mouse CD14 monoclonal antibodies have been raised (23). We feel it is premature to approach these workers with a view to establishing a collaboration until we have raised anti-mouse ELAM-1 monoclonals and verified their performance as immunotoxins.

**c) Synthesis and cytotoxicity testing of
anti-ELAM-1 immunotoxins**

Immunotoxins directed against murine ELAM-1 will be constructed by cross-linking monoclonal anti-mouse ELAM-1 antibodies (as characterized above) to dga using SMPT. The procedure involved will be identical to that described in the previous sections. Activity will be assessed by toxicity studies with cytokine-activated SVEC cells.

**d) Confirmation of ELAM-1 induction on tumor
vasculature and not on normal vasculature**

C3H/He mice bearing 1.0-1.3 cm MM102 or MM48 tumors will be injected i.v with anti-Ly6A.2/anti-CD14 bispecific antibody or with various control materials including unconjugated anti-Ly6A.2 and anti-CD14 antibodies (Fab' and IgG) and diluent alone. Tumors will be removed at various times and cryostat sections will be cut and stained with rat monoclonal antibodies to murine ELAM-1, using standard indirect immunoperoxidase techniques (83). The presence of the bispecific antibody on tumor cells will be verified by staining for rat

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immunoglobulin. Resident macrophages and infiltrating monocytes will be detected by indirect immunoperoxidase staining with anti-Mac-1 (CD 11b/CD 18) antibodies. Cytokine-producing cells will be identified in serial
5 cryostat sections of tumors by *in situ* hybridization with ³⁵S-labeled antisense asymmetric RNA probes for murine IL-1 β and TNF α mRNA, as previously described (84).

e) Tumor therapy experiments.

10

C3H/He mice bearing 1.0-1.3 cm MM102 or MM48 tumors will be injected with 50-100 μ g anti-Ly6A.2/anti-CD14 bispecific antibody or with various control materials including unconjugated anti-Ly6A.2 and anti-CD14
15 antibodies (Fab' and IgG) and diluent alone. One to three days later, the mice will receive intravenous injections of anti-ELAM-1 immunotoxin, an isotype-matched immunotoxin of irrelevant specificity or unconjugated anti-ELAM-1 antibody. Anti-tumor effects will be
20 monitored by measuring the size of the tumors at regular intervals and by histological examination, as in the preceding examples.

C. VERTEBRATE ANIMALS

25

1a) Tumor experiments:

Mice will be injected subcutaneously with 10⁶ MM102 or MM48 tumor cells (in 0.1 ml saline) either on the
30 abdominal wall or on the flank. In some experiments, cyclosporin A (60 mg/kg/day) will be injected intraperitoneally or given in the drinking water. The mice will be observed daily thereafter and the dimensions of the tumor will be measured. When the tumor reaches a
35 diameter of 1.0-1.3 cm, the mice will receive an injection of bispecific antibody (0.1 ml in saline) into

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a tail vein and then 2-3 days later will receive an intravenous injection of immunotoxin, again into the tail vein. The experiment is terminated by euthanizing the mice when their tumors reach 1.5-2 cm in diameter in any dimension.

Each experimental group will comprise 8-10 animals, and there will generally be 5 or 6 treatment groups making a total of 40-60 mice per experiment. One such experiment will be performed per month.

1b) Raising monoclonal antibodies:

Adult Wistar rats will be used. Rats will be immunized by injecting them i.m with mouse endothelial cells (SVECs) homogenized in 0.1 ml of a 50:50 mixture of Freund's incomplete adjuvant and saline. Rats will be boosted 1 month and 2 months later in the same manner. 7-10 days after the second boost, 0.1 ml blood will be removed from tail vein and the serum will be analyzed for the presence of antibody. If sufficiently positive, the rats will be given a final i.m boost with SVEC cells and 3 days later, the rats will be euthanized their spleens dissected out for monoclonal antibody production.

25

1c) Raising ascites:

BALB/c nude mice will be injected intraperitoneally with 0.5 ml Pristane (2, 6, 10, 14-tetramethylpentadecane) 2 to 4 weeks before being injected intraperitoneally with rat hybridoma cells. The mice will be weighed daily and euthanized when their body weight increases by 20% or more due to hybridoma growth in the peritoneal cavity. The contents of the peritoneal cavity will then be drained and monoclonal antibodies purified from the ascitic fluid.

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2. Choice of species and number of animals

Mice: The antitumor effects of immunotoxins in animals cannot be predicted from tissue culture experiments. Such factors as hepatic entrapment, blood clearance rates and binding to serum components makes it essential that intact animal systems are used for evaluation. The choice of mice as the test animal is determined by the fact that inbred strains exist in which mammary tumors will grow reproducibly. The number of animals (8-10) per treatment group is the minimum for statistically significant differences between different treatment groups to become apparent. The number of treatment groups (5-6) per experiment is the minimum for an effect of a specific immunotoxin to be distinguished from an effect of its components (antibody alone, ricin A chain alone, or mixtures of the two) and for superiority over control immunotoxin of irrelevant specificity to be demonstrated.

20

Rats: Since antibodies are to be raised to mouse endothelial cell antigens, it is best to use another species for immunization. Rats are preferred for these studies because they are inbred and respond consistently to the immunogen.

25

EXAMPLE IV

Preparation of Tumor Endothelial Cell Targeted Immunological Reagents

30

The TEC4 and TEC11 monoclonal antibodies were raised and selectively screened in the following manner. HT-29 human colonic adenocarcinoma cells were obtained from the Imperial Cancer Research Fund Central Tissue Bank and seeded at 25% of confluent density in tissue culture flasks in Dulbeccos Modified Eagles Medium (DMEM)

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supplemented with 10% v/v fetal calf serum (FCS), 2.4 mM L-glutamine, 200 units/ml penicillin and 100 µg/ml streptomycin. The cells were allowed to grow to full confluence over 4-5 days incubation at 37°C in a humidified atmosphere of 90% air/10% CO₂ before the supernatant tissue culture medium (hereafter referred to as HT-29 tumor-conditioned medium, HT-29 TCM) was removed, filtered through a 0.22 µm filter to ensure sterility and to remove any particulate matter, and stored at 4°C for no more than one week before use. HT-29 human adenocarcinoma cells were used to prepare TCM because they had previously been shown to secrete angiogenin into their culture medium (Rybak et al., 1987) and angiogenin has been found to induce neovascularization (i.e., profound alteration of endothelial cell behavior) in an *in vivo* assay (Fett et al., 1987).

Human umbilical vein endothelial cells (HUVEC) were incubated in Medium 199 supplemented with 20% w/v FCS, glutamine and antibiotics and mixed 1:1 with HT-29 TCM. After 48 hours at 37°C the endothelial cells were harvested non-enzymatically and $1 - 2 \times 10^6$ cells were injected intraperitoneally into a BALB/c mouse. This entire procedure was repeated three times at two week intervals, the final injection being by the intravenous route. Three days later, splenocytes from the immunized animal were fused with SP2/O murine myeloma cells at a ratio of 1:2 using PEG2000 (Kohler and Milstein, 1975). The cell mixture was introduced into the wells of 96-well flat bottomed microtiter plates along with 3×10^4 syngeneic peritoneal feeder cells per well. Twenty-four hours later 100 µl of medium containing hypoxanthine, ammoterpin and thymidine (HAT Medium) was added to select for fused cells (hybridomas). The cultures were fed with additional HAT Medium at 3 day intervals.

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When hybridomas had grown to high density, 50 μ l samples of supernatant were taken and screened by galactosidase anti-galactosidase (GAG) ELISA (Burrows et al., 1991) for antibodies reactive with HT-29-activated HUVEC. All positive wells were seeded into 24-well plates, expanded by further culture in HAT medium, and retested 7-10 days later by the same technique. All positive wells were harvested and samples stored in liquid nitrogen. The remaining cells from each positive well were cloned in 96 well plates by the limiting dilution method (Kohler and Milstein, 1975). When the clones had grown to high density, 50 μ l samples of supernatant were taken and assayed by GAG ELISA against HT-29 TCM-activated HUVEC and 'resting' HUVEC grown in the absence of tumor-derived factors. Any wells which showed significantly greater reactivity with HT-29 TCM-activated HUVEC than with control HUVEC were recloned and expanded to culture flasks to provide adequate supernatant for additional screening by immunohistochemistry.

Supernatants from these expanded clones were screened by standard indirect immunoperoxidase techniques (Billington and Burrows, 1987) by sequential incubation with F(ab)₂ sheep anti-mouse IgG (1:200, Sigma) and streptavidin-biotin-horseradish peroxidase complex (1:100, Dakopatts) against a small panel of normal and malignant human tissues on cryostat sections. After this round of screening two clones, 1G4 (TEC4) and 2G11 (TEC11), were selected for further study on the basis of significantly greater reactivity with endothelial cells in sections of solid tumors than with those in sections of normal tissues. The 1G4 (TEC4) antibody was found to exhibit strong staining of both large and small vessels in a breast carcinoma, which staining at high power, was seen to be clearly associated with endothelial cells. In

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contrast, a section of a non-malignant breast lesion stained on the same occasion with 1G4 (TEC4) showed no reactivity with vasculature. This was not because there were no blood vessels in the section, because an adjacent
5 section stained with a positive control anti-endothelial cell antibody revealed many brightly-stained structures.

The GAG ELISA was once again employed to characterize which biologically active proteins were
10 capable of mediating induction of the TEC4/TEC11 antigen(s). HT-29 TCM, epidermal growth factor (EGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were found to significantly increase the levels of 2G11 (TEC11) antigen in HUVEC (Figure 13). In contrast,
15 platelet-derived growth factor (PDGF), granulocyte colony-stimulating factor (G-CSF), basic fibroblast growth factor (bFGF), interferon- γ (IFN γ), tumor necrosis factor- α (TNF α), interleukin- 1β (IL- 1β) and FCS alone displayed no significant inducing activity.
20 Similar results were obtained with 1G4 (TEC4). Both EGF (Stoschek and King, 1986) and GM-CSF (Metcalf and Nicola, 1985) can be produced by tumor cells.

The 1G4 (TEC4) and 2G11 (TEC11) antibodies were then
25 screened extensively, by immunohistochemistry as described above, on a large panel of malignant, inflamed and healthy human tissues. The results of these assays are tabulated in Table IV and are depicted in graphic form in Figures 14 and 15. From the large body of data
30 collected, the inventors were able to conclude that (i) the patterns of reactivity of the two antibodies were similar enough to suggest that both reacted with the same antigen and (ii) both antibodies showed a marked selectivity for tumor endothelium, giving strong to very
35 strong staining of endothelial cells in most tumors but weak to no staining of vessels in healthy normal tissues.

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This dramatic difference is particularly well illustrated in Figure 16, where malignant and normal gut tissues from the same patients were stained. A large differential of staining was seen with both antibodies except where the non-malignant sample was chronically inflamed. Further experiments have shown that the 1G4 (TEC4)/2E11 (TEC11) antigen migrates as a doublet of apparent molecular weight 43-45 kilodaltons on non-reducing SDS gels, is trypsin sensitive and is located on the cell surface of activated HUVEC as indicated by indirect immunofluorescence and FACS analyses.

TABLE IV
TABLE OF REACTIVITIES

	A	B	C	TEC4		TEC11		F	G
				1G4	2G11	anti-1CAM	anti-ELAM		
80	VASCULAR EC OF NORMAL	no. sections	FV111	1G4	2G11	anti-1CAM	anti-ELAM		
81	TISSUE TYPE:								
82	Placenta	1:01	ALLV+++	M, A & F, V+-	M&F++	M&F+++	--		
83	Stomach muscle	1:01	C/V, V+++	--	C/V, V+	C/V++	--		
84	Stomach mucosa	1:01	C/V, V+++	C/V+-, V, LU+-	C/V, V+	V+-	C/V, V+		
85	Lung	3:03	ALLV+++	--	C/V, V+-	V+, A-	S, C/V+-		
86	Prostate	1:01	ALLV+++	(V&S, C+) LU, A-	S, V&LU, C/V	C/V+++ V+-, A	S, C/V&+LU, V+		
87	Spleen	1:01	ALLV+++	C/V++, A+-	C/V++, A+-	C/V+++ A+	--		
88	Muscle in leg	1:01	ALLV+++	C/V, V+	C/V, V++	C/V, V+	--		
89	Breast	3:03	ALLV+++	C/V, V+-, A+	C/V, V+, A-	ALLV++	S(C/V&V) -		
90	Colon	1:01	ALLV+++	ALLV+-	ALLV+-	C/V, V++	S(C/V&V) -		
91	Recta-Sigmoid	1:01	ALLV+++	ALLV-(1 C/V=++)	C/V, V++	C/V, V+++ A	--		
92	Caecum	1:01	ALLV+++	C/V-, V+-- A-	C/V&V+-, A+-	C/V+++ V+-, A-	S, C/V+		

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Table IV - continued

	A	B	C	D	E	F	G
93	Thymus	1:01	C/V,V	S(C/V,V)+--	C/V, V++	C/V+	V+--
94	Kidney Cortex	1:01	ALLV+++	C/V+--	C/V, A+--	C/V+--	--
95	Liver	1:01	ALLV+++	V+--	V+, A+--	A+--	--
96	Pancreas	1:01	ALLV+++	--	ALLV+--	C/V+++ , V&A+	S, V+--
97	INFALMED TISSUES:						
98	Reactive Hyperplasia	1:02	ALLV+++	S(C/V,V)+	C/V, V+	C/V, V++	S(C/V,V)+
99	Cat Scratch Fever (reactive)	1:01	ALLV+++	C/V,V+	C/V, V++	ALLV(LA+++ ,NLA+)	ALLV(LA+ ,NLA+)
100	Colon: Ulcerative Colitis	1:02	ALLV+++	C/V,V+	C/V, V+++ , A +	ALLV+	S, V+
101	Sigmoid: Ulcerative Colitis	1:01	ALLV+++	(LAC/V+++) C/V+, V+	(LAC/V+++) ALLV+	ALLV+	--
102	Tonsil	3:04	ALLV+++	C/V, V++	C/V, V+++ , A +	C/V, V+++ , A ++	S(C/V,V)+
103							

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Table IV - continued

	A	B	C	D	E	F	G
104	NON-VASC' STAINING OF NORMALS:		TEC11				
			TEC4				
105	TISSUE TYPE:	no. sections	FV111	1G4	2G11	anti-1CAM	anti-ELAM
106	Placenta	1:01	--	PFV1, PMSE+	PFV1, PMSE++	--	--
107	Stomach muscle	1:01	--	SB++ , SM++	SB++	SB++	SB+
108	Stomach mucosa	1:01	--	CLN	--	LT++	--
109	Lung	3:03	--	--	--	AL+	--
110	Prostate	1:01	--	FBST++	FBST++	ISLETS OF CLS++	--
111	Spleen	1:01	--	RED PULP++	RED PULP+++	GENERALLY++	--
112	Muscle in leg	1:01	--	--	--	--	--
113	Breast	3:03	--	ME+++	--	BMME+- , LT+++	S, C+-
114	Colon	1:02	--	ST+ , SM+-	ST+	FB, LT++	--
115	Recto-Sigmoid	1:01	--	CLM&SM++	ST+-	--	--
116	Caecum	1:01	--	ST&SM+-	ST+ , S, LE+ +	LT+++	--
117	Thymus	1:01	--	--	--	LE+++	--
118	Kidney Cortez	1:01	--	TU++ , ST+	CT+++	GL, CT, LE++ +	--
119	Liver	1:01	--	BD&S, HP+	ST+ , SIM++	HP+++	--
120	Pancreas	1:01	--	SM+++ , ST+-	SM+++ , ST+ -	SM+++ , ST+-	--

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Table IV - continued

	A	B	C	D	E	F	G
121	INFLAMED TISSUES:						
122	Reactive Hyperplasia	1:02	--	--	LE++	LE+++	--
123	Cat Scratch Fever (reactive)	1:01	--	--	--	CLN+++	--
124	Colon: Ulcerative Colitis	1:02	--	GCLS, SM++	GCLS+ -, ST+	ALE&LE++	ALE++
125	Sigmoid: Ulcerative Colitis	1:01	--	CLN+, SM+, G EP++	ST+	LE<+++	--
126	Tonsil	3:04	--	CLN++	LNP+	LN+++ +, ALE+	--
127							
128	KEY:						
129	A= Arteries/Arterioles			KE= Keratin			
130	AL= Alveoli			LA= Leucocyte associated			
131	ALE= Adherent leucocytes			LE= Leucocytes			
132	ALLV= All vessels			LN= Lymphoid nodules			
133	AN= Angiosarcoma			LNP= Lymphoid nodule peripheral leucocytes			
134	BCP= Bowman's capsule			LT= Lymphoid tissue			
135	BD= Bile ducts			LU= Luminal			
136	BM= Basement membrane			M= Maternal			

Table IV - continued

	A	B	C	D	E	F	G
137	C/V= Capillaries/Venules			ME= Myoepithelial cells			
138	CE= Columnar epithelium			NE= Nerve		Very strong = +++	
139	CLN= Leucocytes at core of lymphoid nodules			NLA= Non-leucocyte associated		Moderate to strong = ++	
140	CLS= Cells			NT= Necrotic tumour		Moderate = +	
141	CO= Collagen			NTA= Non-tumour associated		Weak = +-	
142	CP= Capsule			PFV1= Periphery of fetal vi		Very weak & faint = +-	
143	CT= Connective tissue			PMSE= Periphery of maternal septa		Negative = +-	
144	CD= Ducts			S= Selectivity positive			
145	ED= Epidermis			SB= Submucose		#= patient 1	
146	EL= Elastin			SCE= Statified Cuboidal Epithelium		#= patient 2	
147	EP= Epithelium			SIN= Sinusoidal cells		#= patient 3	
148	F= Foetal			SK= Skin			
149	FB= Fibromuscular			SKM= Skeletal muscle			
150	G= Gland			SM= Smooth muscle			
151	FCLS= Gland cells			ST= Streoma			
152	GEP= Glandular epithelium			TA= Tumour associated			
153	GL= Glomeruli			TU= Tubules			
154	HP= Hepatocytes			TUM= Tumour cells			

Table IV - continued

NOTES FOR TABLE IV

No. sections: This value shows the number of sections stained which correlate with the data shown in the table. The remaining difference did not correlate with the data.

Capillaries/Venules: These are classified together since they are almost indistinguishable from each other in stained section.

FVIII = Used at 1/20 dilution in serum free RPMI.

IG4 & 2G11 = Both used as neat culture supernatants containing 10% FCS. (i.e.: TEC4, TEC11)

ICAM & ELAM = Both used at 1/2 dilution of culture supernatants in serum free RPMI.

Caecum carcinoma # Both these sections are taken from the same patient no 1

Sigmoid carcinoma # Both these sections were taken from the same patient no. 2

Sigmoid ulcerative colitis #

Recto-sigmoid carcinoma ∞ Both these sections were taken from the same patient no. 3

Normal Recto-sigmoid ∞

ABBREVIATIONS

Example 1). S(C/V, V) +---.

Reads as: Selected capillaries/venules & veins stain +--- or very faintly.

Example 2). ALLV - (1C/V = ++).

Reads as: All vessels negative (one capillary/venule stains ++ or moderate to strong).

Example 3). S, V & C/V, LU +++/

Reads as: Selected veins & capillary lumens stain +++ or strong.

Table IV - continued

GENERAL

1G4 & 2G11 (TEC4 & TEC11) stain tumor vascular endothelium more strongly than normal or benign vascular endothelium, and seem to bind an antigen which is up-regulated in tumors. However, vessels associated with leukocytes or lymphoid tissue in tumors and in inflamed tissues, also show stronger staining than in normal or benign tissues.

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CLAIMS

1. The use of an antibody or antibody fragment that
5 recognizes a cell surface antigen associated with
endothelial vasculature of a vascularized tumor mass, the
antibody or fragment linked to a selected therapeutic or
diagnostic agent, in the preparation of a medicament for
the treatment of vascularized tumors.
- 10
2. A use according to claim 1, wherein the antibody is
linked to a paramagnetic, radioactive or fluorogenic ion
that is detectable upon imaging.
- 15
3. A use according to claim 2, wherein the antibody is
linked to a paramagnetic ion selected from the group
consisting of chromium (III), manganese (II), iron (III),
20 iron (II), cobalt (II), nickel (II), copper (II),
neodymium (III), samarium (III), ytterbium (III),
gadolinium (III), vanadium (II), terbium (III),
dysprosium (III), holmium (III) and erbium (III).
- 25
4. A use according to claim 3, wherein the antibody is
linked to a radioactive ion, selected from the group
consisting of iodine¹²³, technetium^{99m}, indium¹¹¹,
rhenium¹⁸⁸, rhenium¹⁸⁶, copper⁶⁷, iodine¹³¹, yttrium⁹⁰,
30 iodine¹²⁵, astatine²¹¹, and gallium⁶⁷.
5. A use according to claim 1, wherein the antibody is
linked to an anticellular agent capable of killing or
35 suppressing the growth or cell division of endothelial
cells.

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6. A use according to claim 5, wherein the anticellular agent comprises a chemotherapeutic agent, radioisotope or cytotoxin.

5

7. A use according to 6, wherein the anticellular agent comprises a steroid, an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic, an alkylating agent or an epipodophyllotoxin.

10

8. A use according to claim 6, wherein the anticellular agent comprises a plant-, fungus- or bacteria-derived toxin.

15

9. A use according to claim 8, wherein the toxin comprises an A chain toxin, a ribosome inactivating protein, α -sarcin, aspergillin, restrictocin, a ribonuclease, diphtheria toxin or *Pseudomonas* exotoxin.

20

10. A use according to claim 9, wherein the toxin comprises deglycosylated ricin A chain.

25

11. A use according to claim 1, wherein the agent comprises a coagulant, a cytokine, growth factor, bacterial endotoxin or the lipid A moiety of bacterial endotoxin.

30

12. A use according to claim 1, wherein the antibody recognizes a tumor vascular endothelial cell antigen that is expressed on the cell surfaces of tumor vascular

35

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endothelial cells to a greater degree than on the surfaces of normal endothelial cells.

- 5 13. A use according to claim 12, wherein the antibody recognizes a cell surface protein that migrates as a doublet at about 43K upon SDS-PAGE.
- 10 14. A use according to claim 12, further comprising preparing the antibody by a method that includes the steps of:
- stimulating endothelial cells with tumor-conditioned
15 medium;
- employing the stimulated endothelial cells as immunogens to prepare a collection of antibody-producing hybridomas;
20
- selecting from the collection a hybridoma that produces an antibody that recognizes the activated vascular endothelium to a greater degree than it recognizes non-activated
25 vascular endothelium; and
- culturing the hybridoma to provide the antibody.
- 30 15. A use according to claim 1, wherein the antibody recognizes an antigen whose expression on the surface of the vascular endothelial cell is induced.
16. A use according to claim 15, wherein the antibody
35 recognizes ELAM-1, VCAM-1, ICAM-1, LAM-1 agent or an MHC Class II antigen.

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activating antibody that recognizes a leukocyte cell surface activating antigen.

- 5 24. A use according to claim 23, wherein the activating antigen is further identified as CD2, CD3, CD14, CD16, FcR for IgE, CD28 or the T-cell receptor antigen.
- 10 25. A use according to claim 24, wherein the cytokine is released by a mature effector cell by means of a cross-linking antibody that recognizes a cell surface antigen of mature effector cells, including CD5, CD8, CD11/CD18, CD15, CD32, CD44, CD45 or CD64, or a leukocyte activation
15 antigen including CD25, CD30, CD54 or CD71.
26. A use according to claim 23, wherein the activating antibody is further defined as a bispecific cross-linking
20 antibody, the antibody capable of cross-linking activated leukocytes to tumor cells of the patient's tumor cell mass.
- 25 27. A use according to claim 26, wherein the cross-linking antibody is further defined as a bispecific antibody that recognizes a human tumor-associated antigen and CD14 or CD28.
- 30 28. A use according to claim 27, wherein the bispecific cross-linking antibody is further defined as recognizing CD14 or CD28, and a human tumor antigen selected from the group consisting of p185^{HER2}, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA), the high Mr
35 melanoma antigens recognized by the 9.2.27 antibody, or

-141-

the ovarian-associated antigens recognized by OV-TL3 or MOV18.

5 29. A use according to claim 1, wherein the expression
of MHC Class II molecules by endothelial cells in the
animal's normal tissues is suppressed with cyclosporin,
and MHC Class II molecules of the tumor associated
endothelial vasculature are induced.

10

30. A use according to claim 29, wherein the tumor
vasculature-associated MHC Class II molecules are induced
by activating Helper T cells, CD8-positive T cells or NK
15 cells in the tumor in a cyclosporin-independent fashion.

31. A use according to claim 1, wherein IFN- γ production
by the animal's T-cells is suppressed by means of an
20 anti-CD4 antibody, and IFN- γ production by tumor cells of
the tumor cell mass is selectively induced by introducing
into the animal's bloodstream an IFN- γ -producing T-cell
clone specific for an antigen expressed by said tumor
cells.

25

32. A use according to claim 1, further comprising
administering an antitumor antibody-agent conjugate to
the animal.

30

33. A use according to claim 32, wherein the antitumor
antibody comprises HMFG-2, SM-3, B72.3, PR5C5, PR4D2,
9.2.27, OV-TL3, MOV18 or anti-p185^{HER2}.

35

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r the ovarian-associated antigens recognized by OV-TL3 or MOV18.

5 29. A use according to claim 1, wherein the expression of MHC Class II molecules by endothelial cells in the animal's normal tissues is suppressed with cyclosporin, and MHC Class II molecules of the tumor associated endothelial vasculature are induced.

10

30. A use according to claim 29, wherein the tumor vasculature-associated MHC Class II molecules are induced by activating Helper T cells, CD8-positive T cells or NK
15 cells in the tumor in a cyclosporin-independent fashion.

31. A use according to claim 1, wherein IFN- γ production by the animal's T-cells is suppressed by means of an
20 anti-CD4 antibody, and IFN- γ production by tumor cells of the tumor cell mass is selectively induced by introducing into the animal's bloodstream an IFN- γ -producing T-cell clone specific for an antigen expressed by said tumor cells.

25

32. A use according to claim 1, further comprising administering an antitumor antibody-agent conjugate to the animal.

30

33. A use according to claim 32, wherein the antitumor antibody comprises HMFG-2, SM-3, B72.3, PR5C5, PR4D2, 9.2.27, OV-TL3, MOV18 or anti-p185^{HER2}.

35

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34. A use according to claim 1, wherein the medicament is intended for administration to a human cancer patient.

5 35. The method of claim 1, wherein the medicament is intended for use in an animal model system.

10 36. The use of an antibody or antibody fragment that recognizes a selectively expressible MHC class II molecule on the cell surface of tumor-associated endothelial vasculature, the antibody or fragment linked to a selected therapeutic or diagnostic agent, in the preparation of a medicament for the treatment of
15 vascularized tumors.

20 37. A use according to claim 36, wherein the recognized MHC Class II molecule is one suppressible through the use of cyclosporin.

25 38. A use according to claim 36, wherein the recognized MHC Class II molecule is one induced through the selective activation of Helper T cells, CD8-positive T or NK cells in the tumor.

30 39. A use according to claim 38, wherein the antibody comprises bispecific antibody recognizing both a CD28 antigen on the surface of the T cells and a tumor antigen on the cell surface of tumor cells of the tumor mass, the bispecific antibody being effective to induce the expression of IFN- γ in the tumor by the T cells.

35

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40. A method for targeting a selected agent to tumor-associated endothelial vasculature in an animal having a vascularized tumor mass, the method comprising the steps of:

5

introducing into the patient's bloodstream an anti-CD4 antibody to suppress IFN- γ production by the animal's T-cells;

10

introducing into the animal's bloodstream an IFN- γ -producing T-cell clone that is specific for a tumor antigen on the surface of tumor cells of the tumor mass;

15

preparing an antibody that recognizes an MHC Class II determinant;

linking the selected agent to said antibody; and

20

introducing the antibody-linked agent into the animal's bloodstream.

41. The method of claim 40, wherein the IFN- γ -producing T-cell clone is prepared by a method that includes:

25

removing from the animal a portion of the tumor mass;

30

extracting tumor infiltrating leukocytes from the tumor; and

expanding the tumor infiltrating leukocytes *in vitro* to provide the IFN- γ producing clone.

35

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42. The method of claim 40, further comprising selecting a high IFN- γ producing T-cell clone from the expanded leukocytes.

5

43. The method of claim 41, wherein the anti-CD4 antibody is an anti-CD4 Fab fragment.

10 44. A method for targeting a selected agent to tumor-associated endothelial vasculature in an animal having a vascularized tumor mass, the method comprising the steps of:

15 introducing into the bloodstream of the animal a bispecific antibody, said bispecific antibody recognizing both an activating antigen on the cell surface of monocyte/macrophage cells and a tumor antigen on the surface of tumor cells of
20 the tumor mass, the bispecific antibody being effective to induce the expression of IL-1 or TNF- α in the tumor by monocytes/macrophages; and

25 introducing into the animal's bloodstream an anti-ELAM-1 antibody linked to the selected agent.

45. An anti-ELAM-1 antibody or antibody fragment linked
30 to a selected therapeutic or diagnostic agent.

46. In combination,

35 a first pharmaceutical composition comprising a bispecific antibody recognizing an activating

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antigen on the cell surface of a leukocyte cell
and a tumor antigen on the cell surface of
tumor cells of a vascularized solid tumor; and

5 a second pharmaceutical composition comprising a
second antibody or fragment thereof linked to a
selected therapeutic or diagnostic agent that
recognizes the induced endothelial antigen.

10

47. The combination of claim 46, wherein the activating
antigen induced by the bispecific antibody is further
identified as CD2, CD3, CD14, CD16, FcR for IgE, CD28 or
the T-cell receptor antigen.

15

48. The combination of claim 47, wherein the bispecific
antibody recognizes CD14, and induces the expression of
IL-1 by monocyte/macrophage cells in the tumor.

20

49. The combination of claim 47, wherein the second
antibody recognizes ELAM-1.

25

50. The combination of claim 46, wherein the bispecific
antibody recognizes CD28, and induces the expression of
IFN- γ by T-cells in the tumor.

30

51. The combination of claim 49, wherein the second
antibody recognizes an MHC Class II antigen.

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52. The combination of claim 51, further including a third pharmaceutical composition comprising cyclosporin A.

5

53. The combination of claim 46, further including a third pharmaceutical composition comprising an antitumor immunotoxin.

10

54. The combination of claim 46, wherein the bispecific antibody recognizes a tumor antigen selected from the group consisting of p185^{HER2}, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA), the high Mr melanoma antigens recognized by the 9.2.27 antibody, or the ovarian-associated antigens recognized by OV-TL3 or MOV18.

20

55. The combination of claim 53, wherein the antitumor immunotoxin recognizes a tumor antigen selected from the group consisting of p185^{HER2}, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA), the high Mr melanoma antigens recognized by the 9.2.27 antibody, or the ovarian-associated antigens recognized by OV-TL3 or MOV18.

25

56. The combination of claim 46, wherein the second antibody or fragment comprises an antitumor endothelial cell immunotoxin.

30

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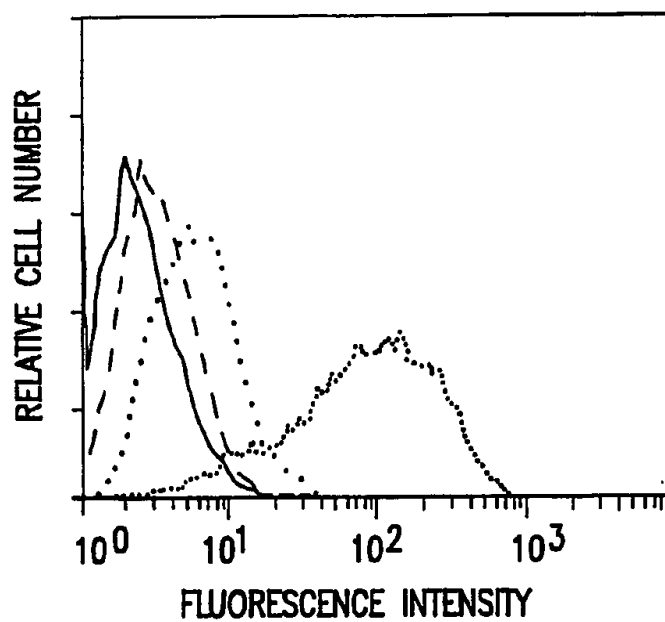


FIG.1A

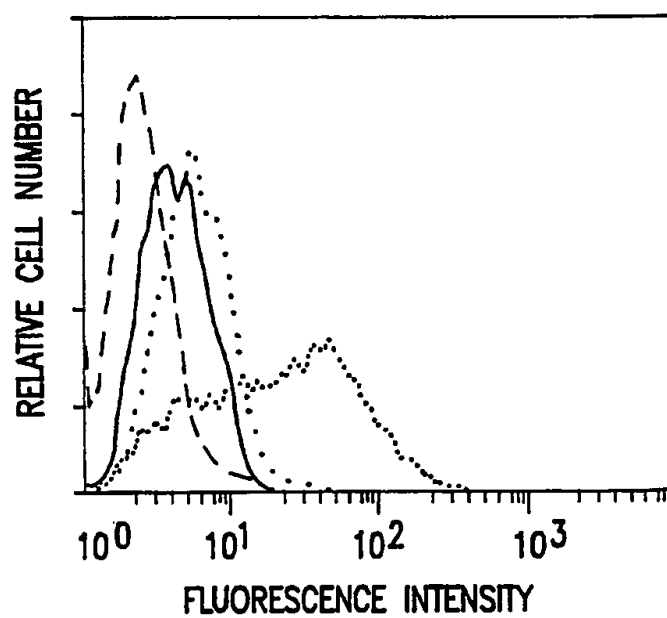


FIG.1B

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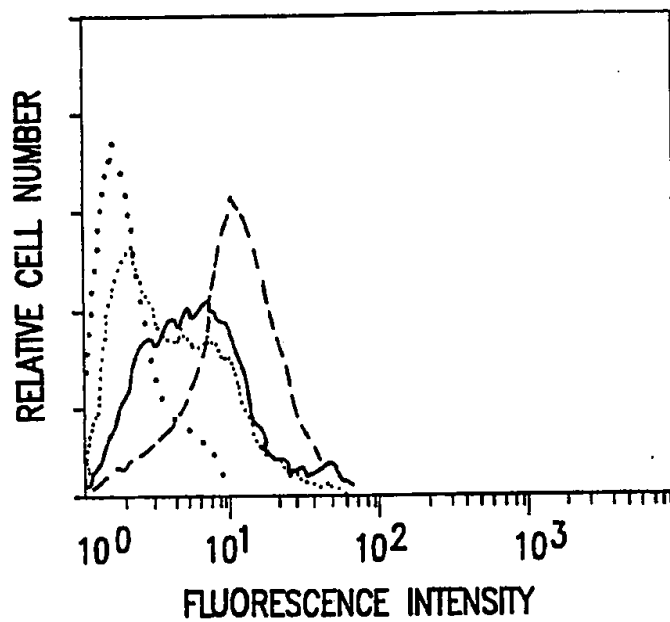


FIG. 2A

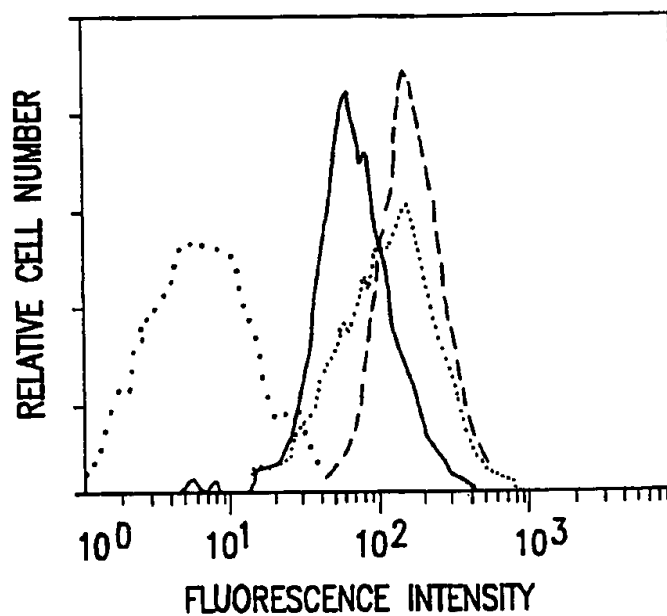


FIG. 2B

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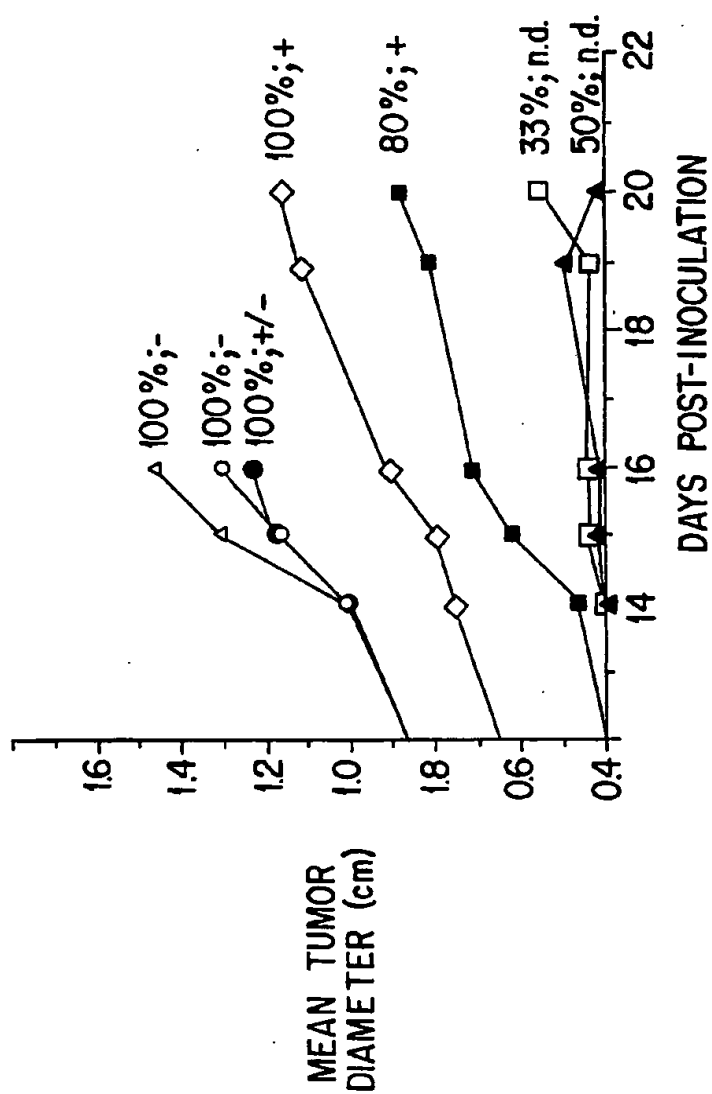


FIG.3

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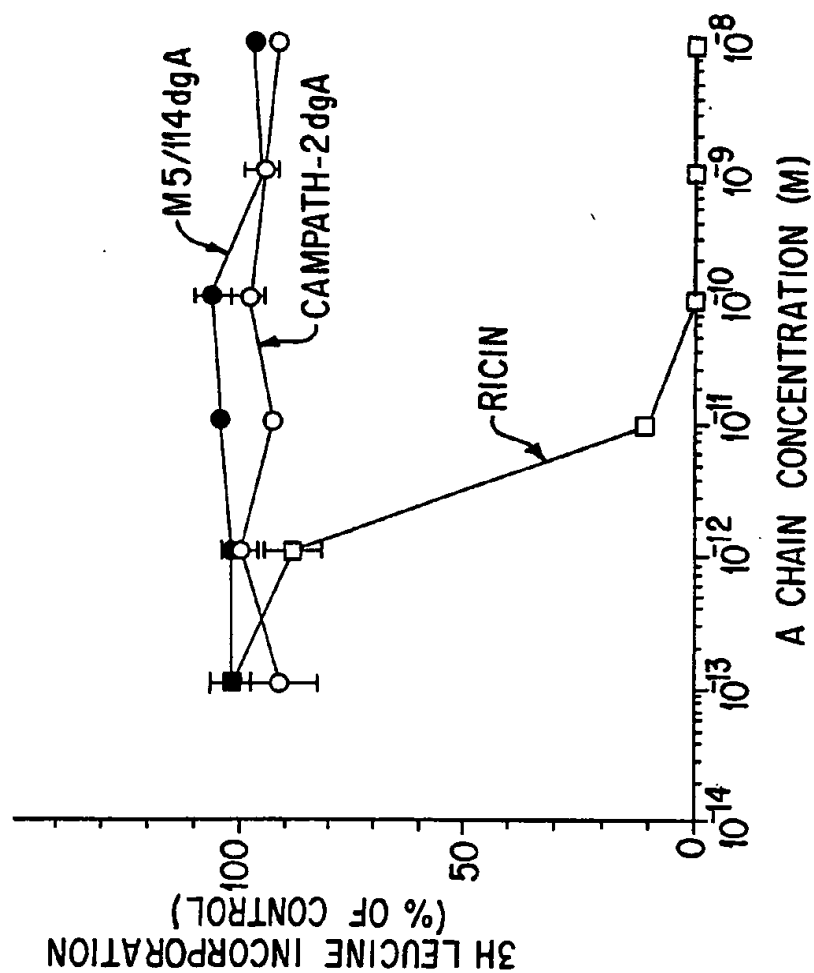


FIG. 4A

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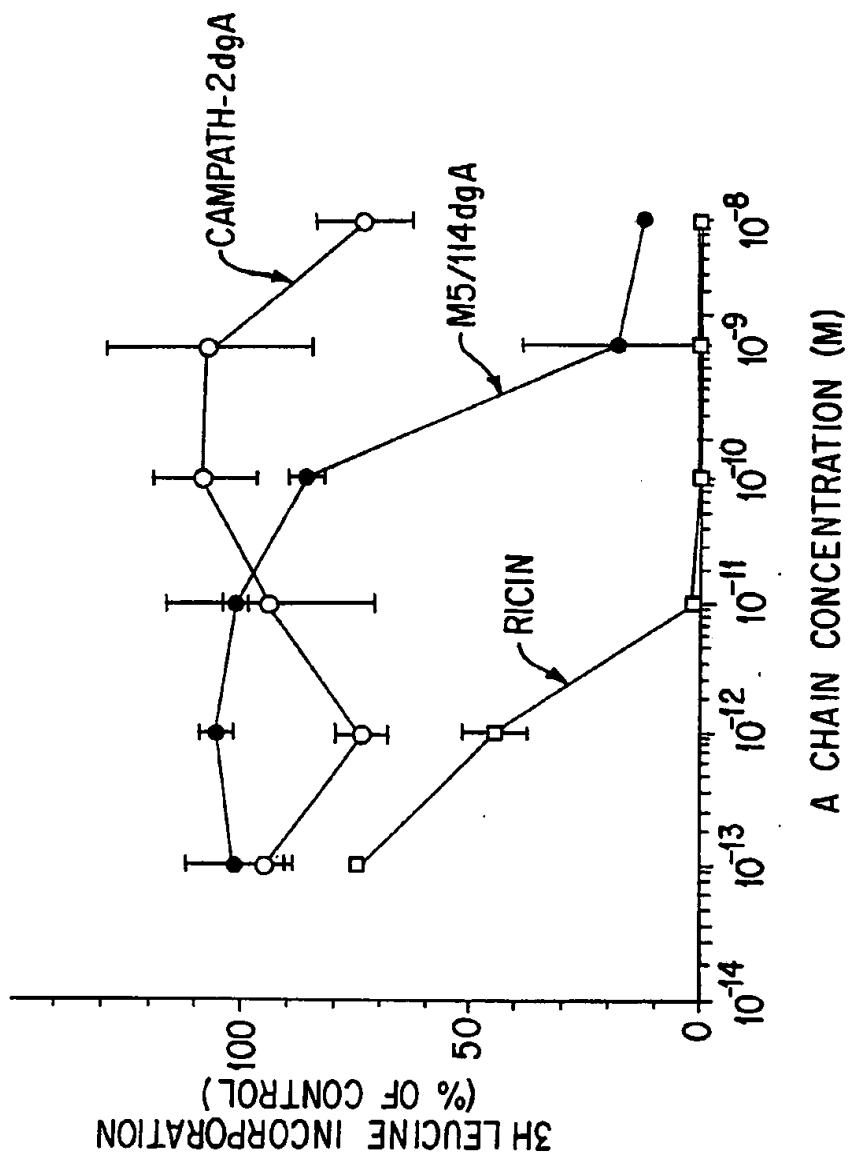


FIG. 4B

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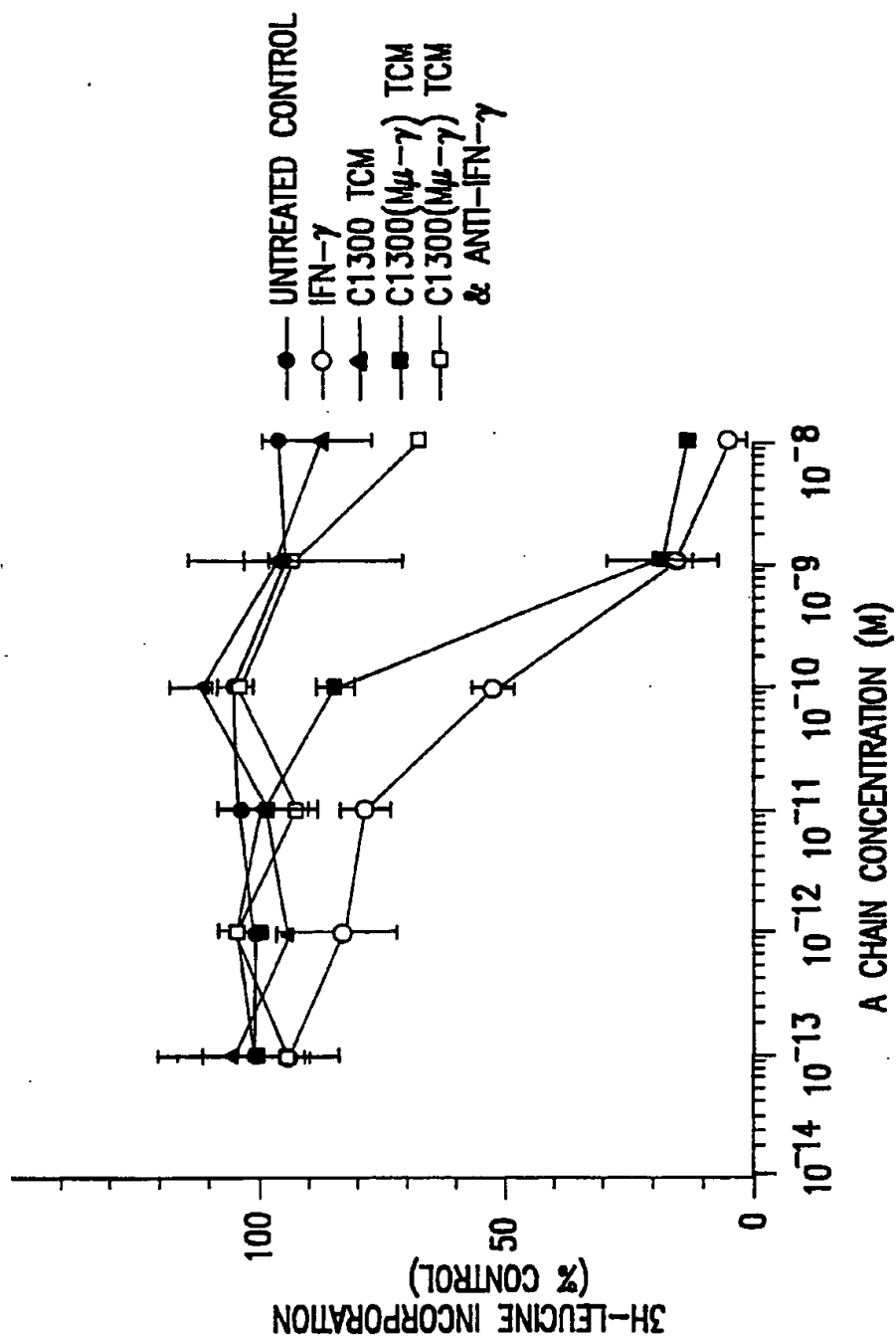


FIG.5

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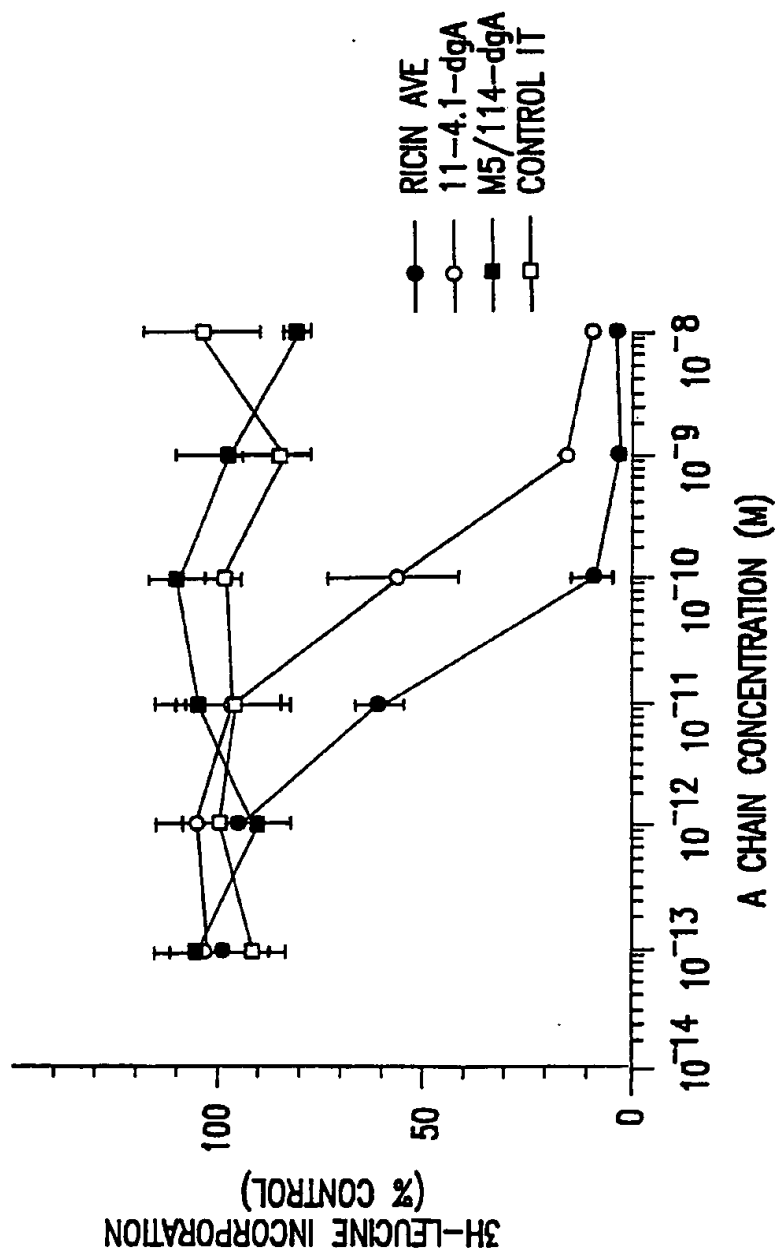


FIG.6A

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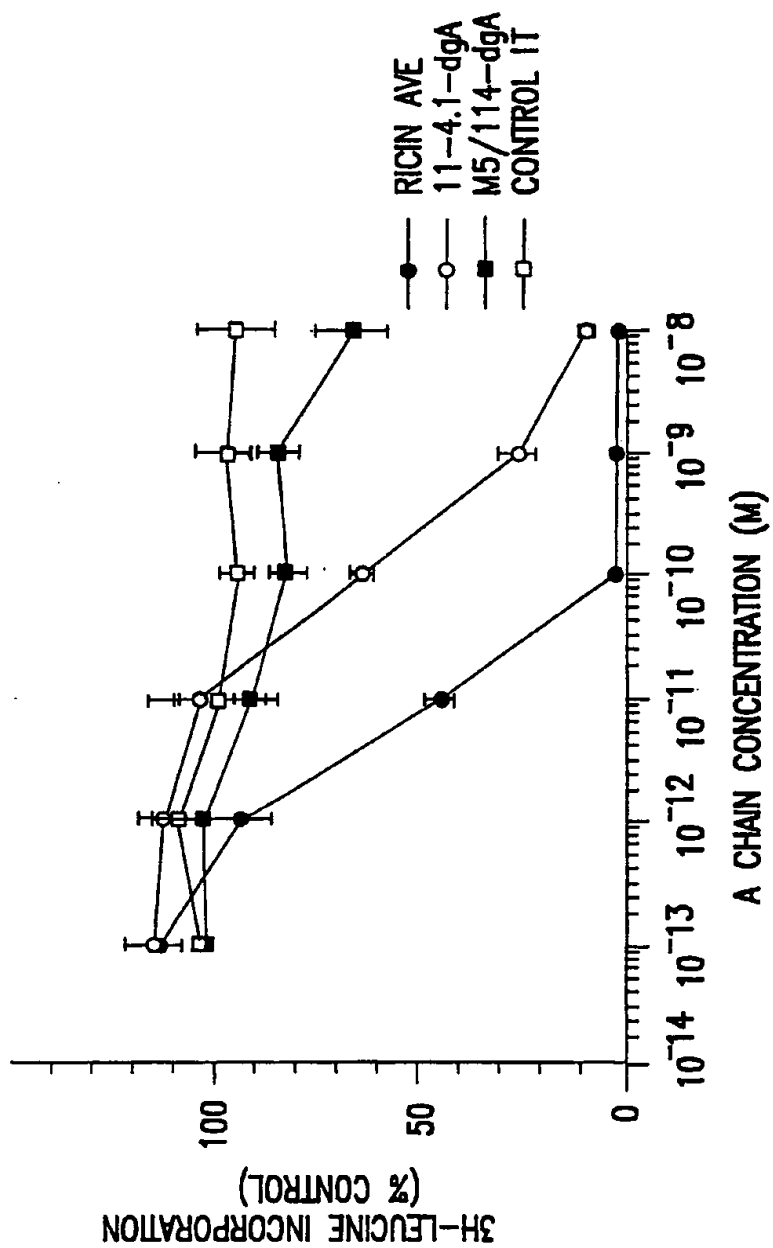


FIG.6B

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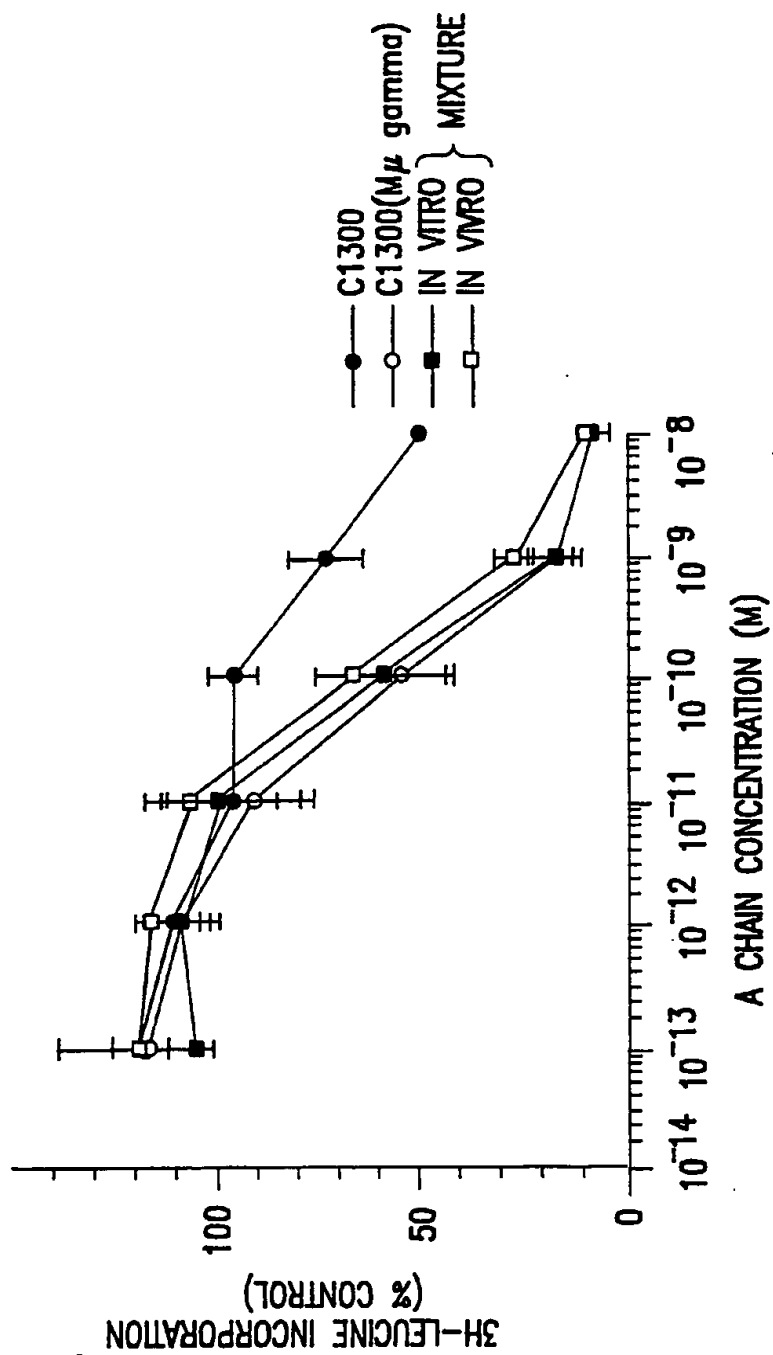


FIG.7A

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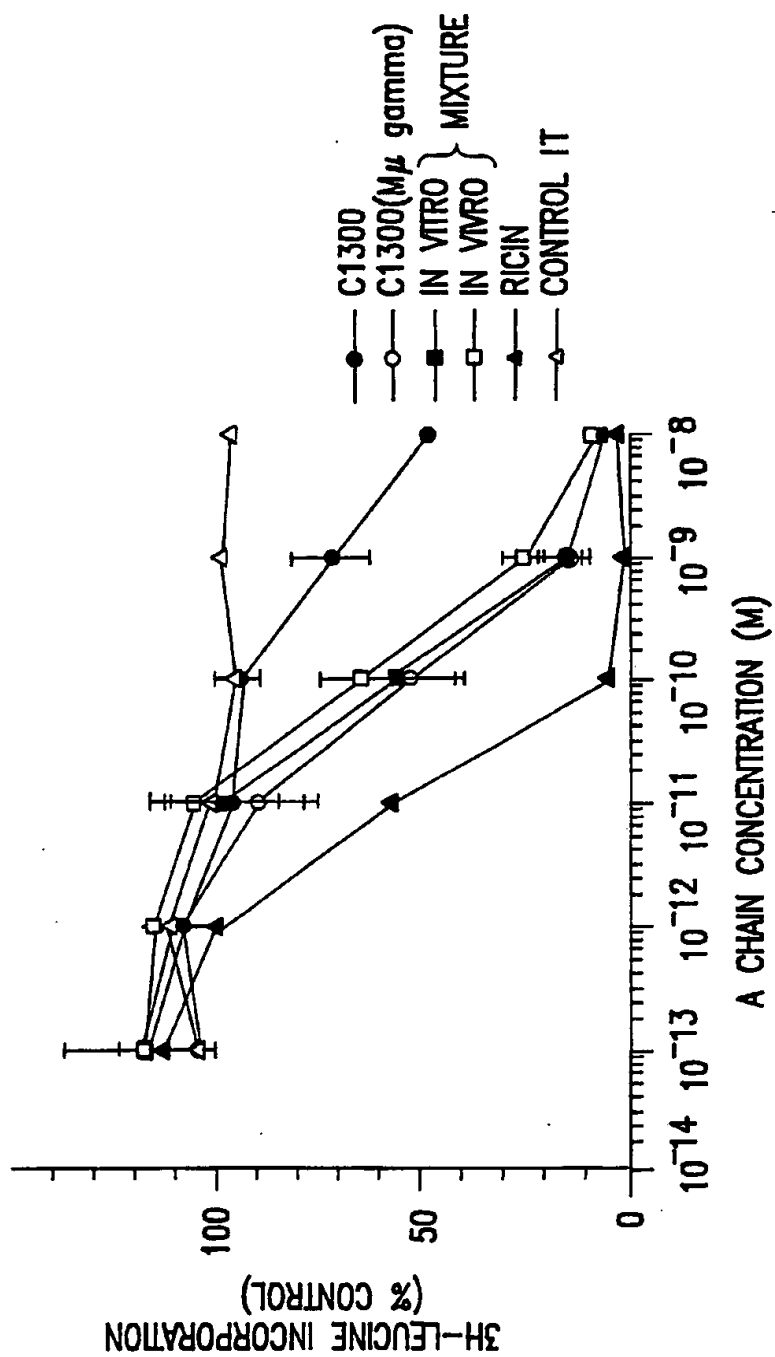


FIG.7B

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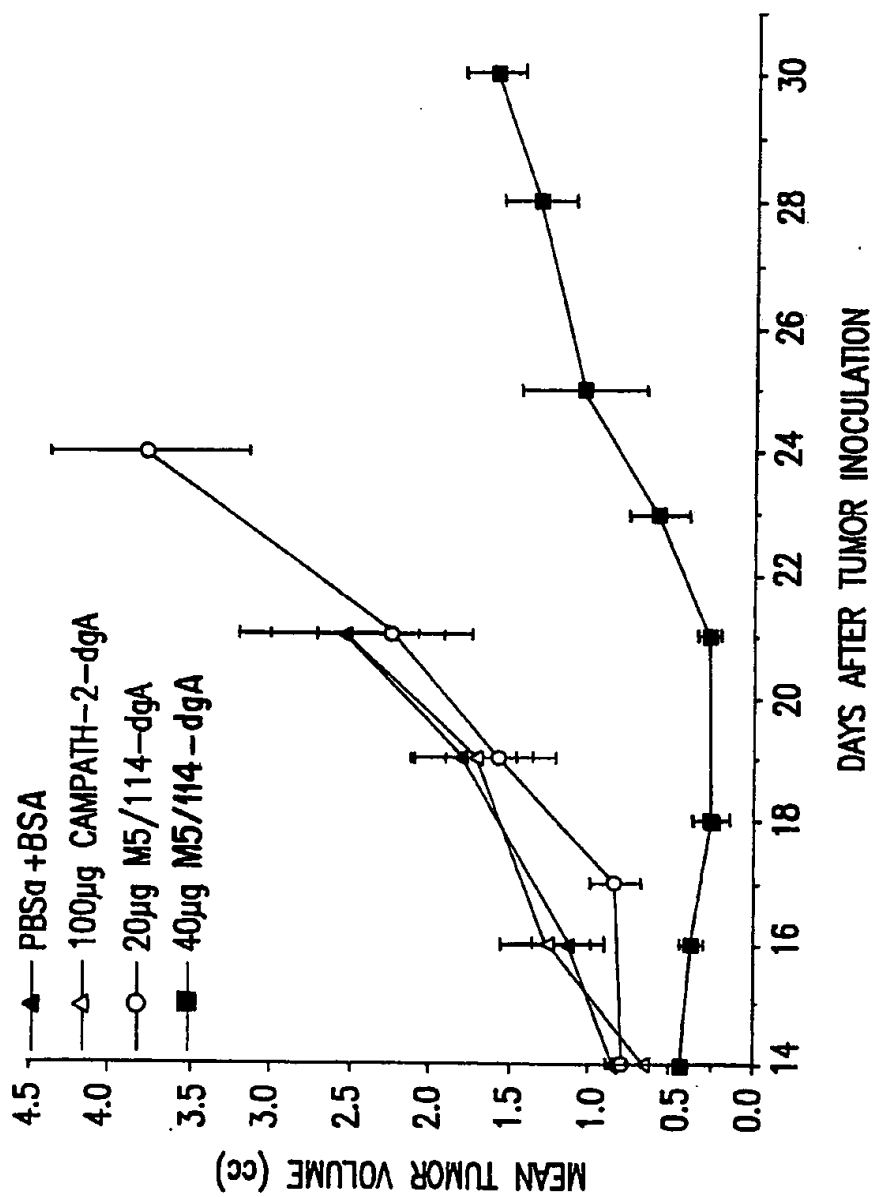


FIG.8

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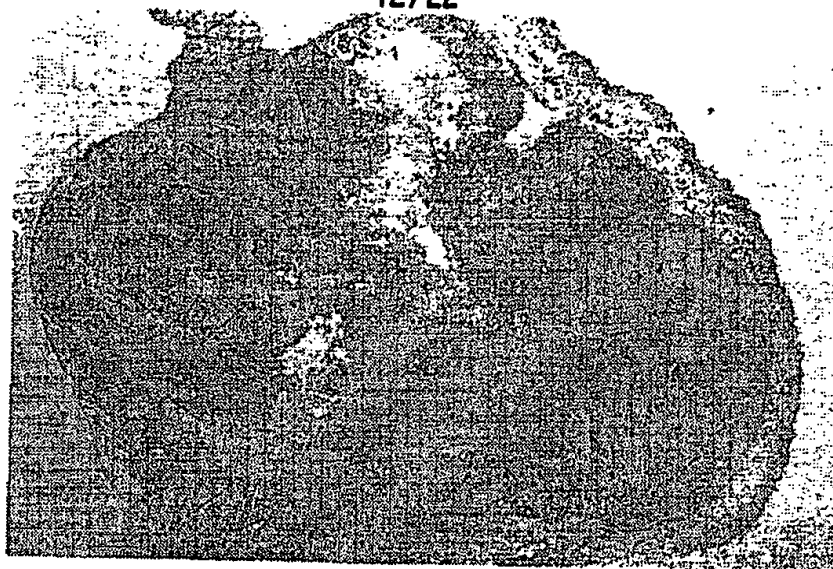


FIG. 9

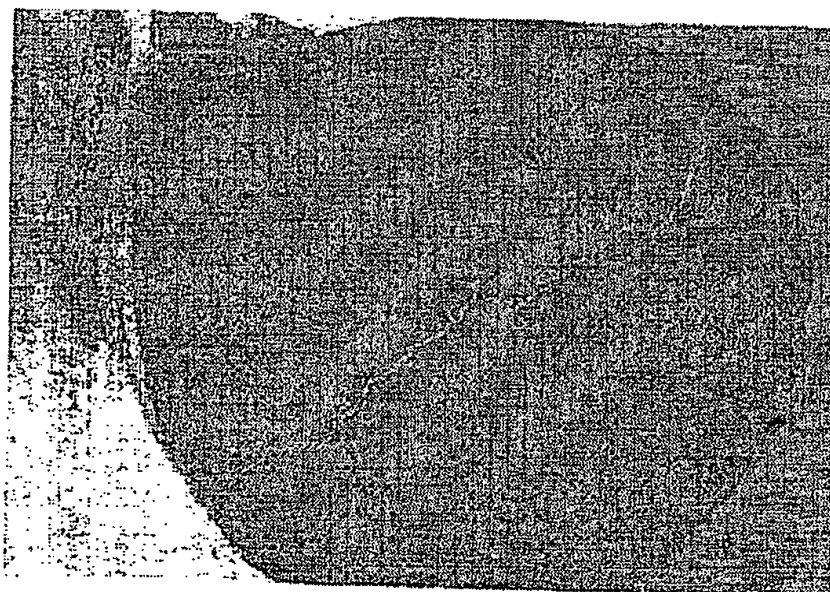


FIG. 10

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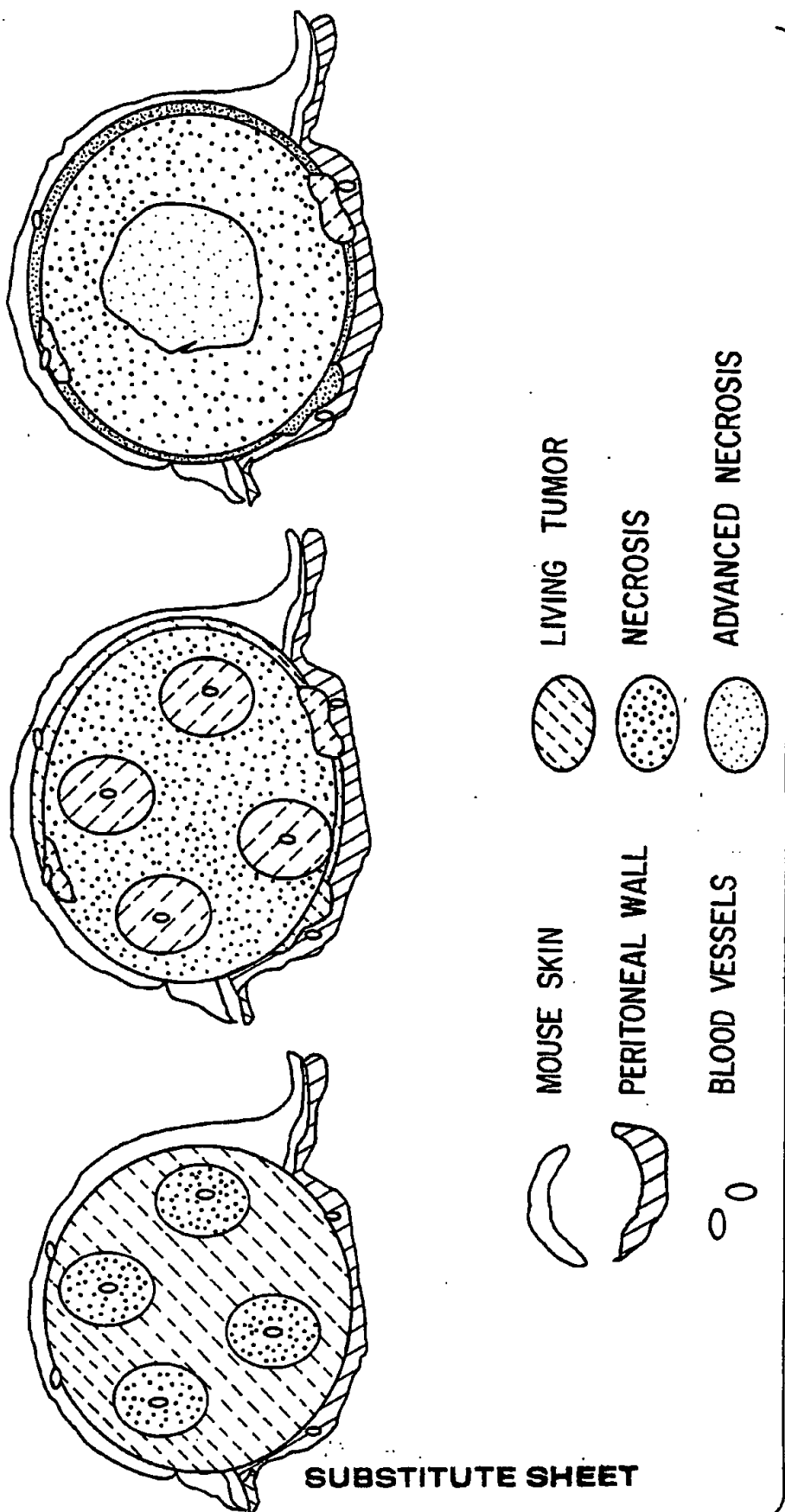


FIG.11

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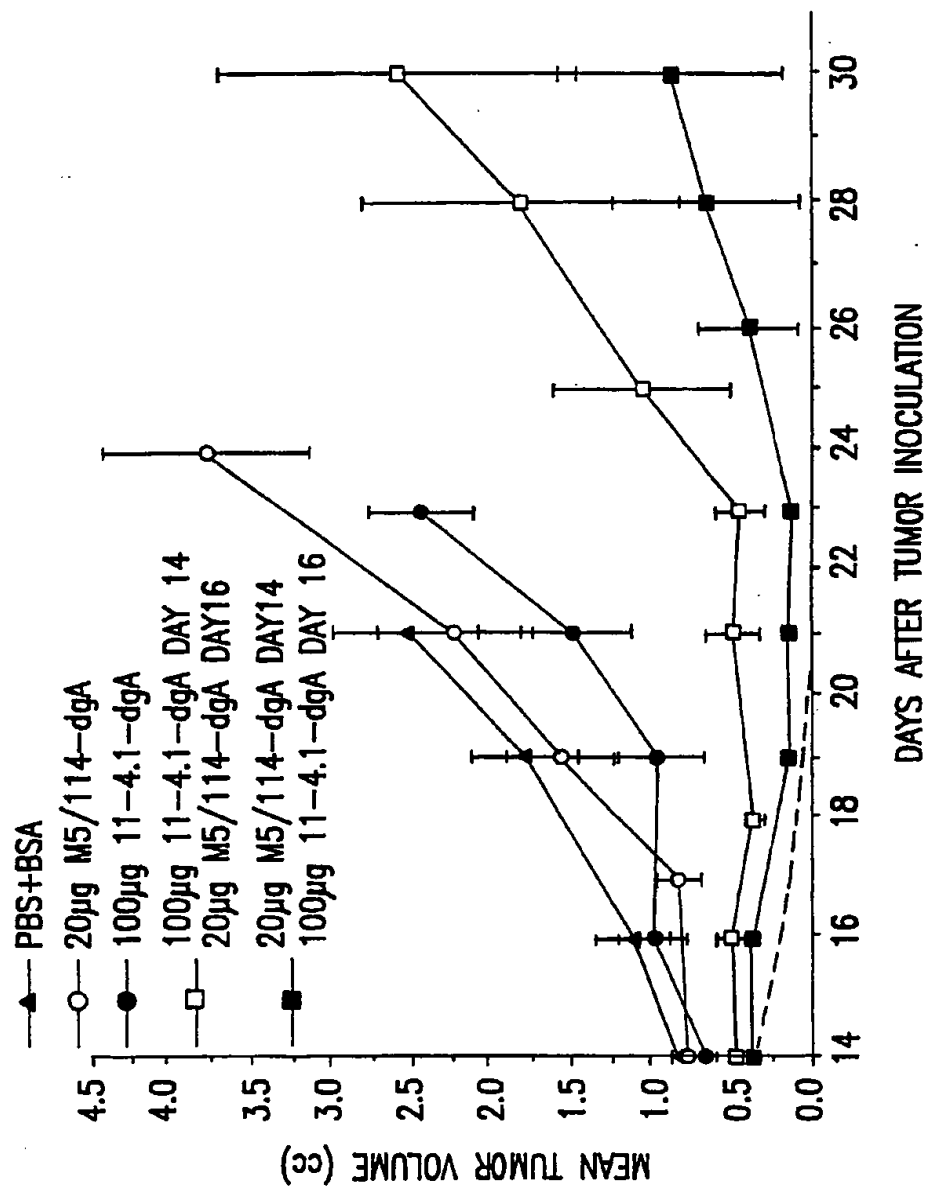
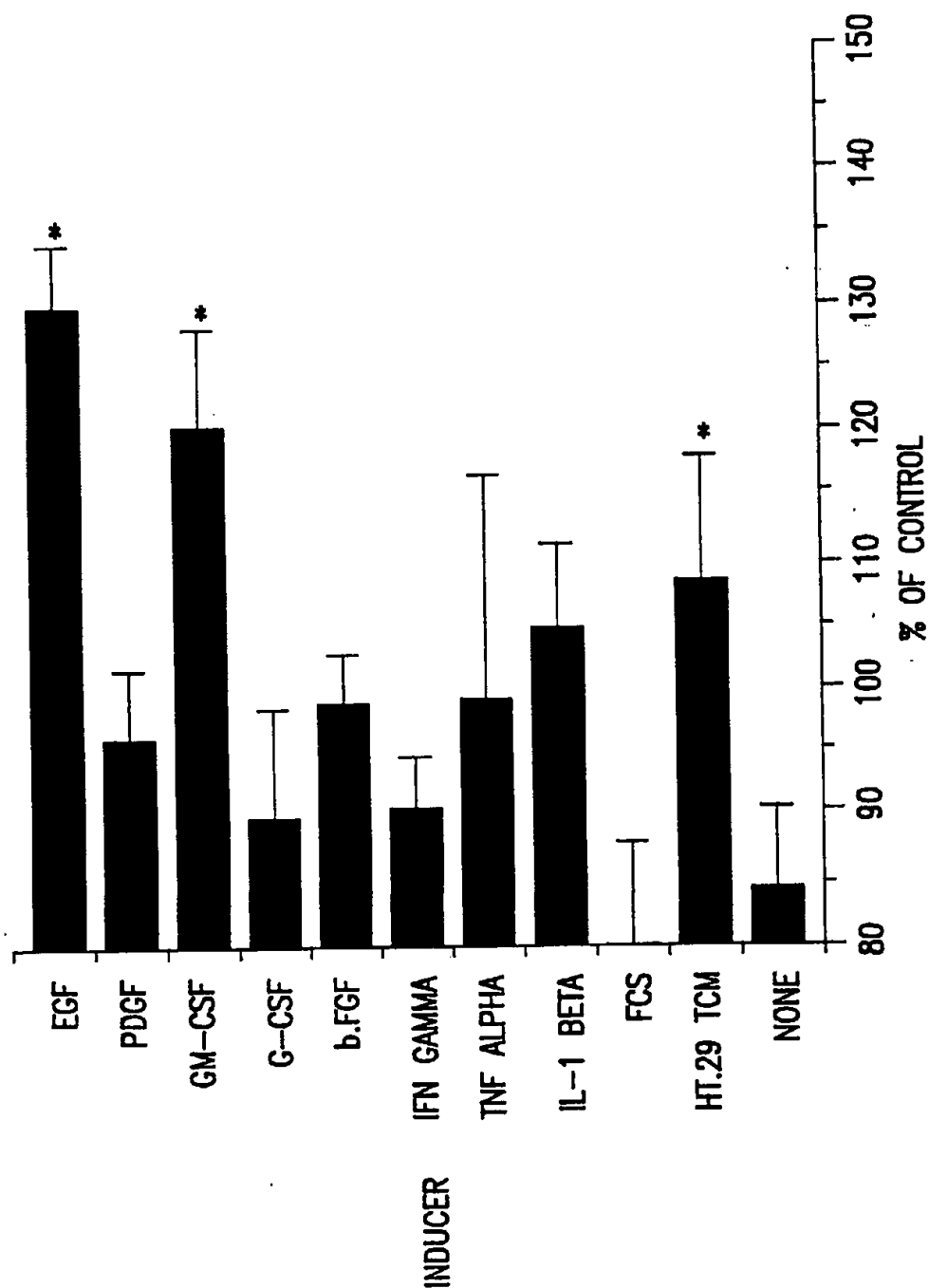


FIG.12

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*: SIGNIFICANTLY HIGHER THAN UNTREATED CONTROL

FIG.13

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		FVIII		1G4		2G11		ICAM		ELAM	
		TA	NTA	TA	NTA	TA	NTA	TA	NTA	TA	NTA
<u>PAROTID TUMOUR</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■	■	▨	▨	▨	▨	▨	▨	▨	▨
VEINS		■	■	▨	▨	▨	▨	▨	▨	▨	▨
ARTERIES / ARTERIOLES		■	■	□	□	□	□	□	□	□	□
<u>MAMMARY CARCINOMA</u>											
NO. SECTIONS: 12/13											
CAPILLARIES / VENULES		■		▨		▨		▨ ^S		▨ ^S	
VEINS		■		▨		▨		▨		□	
ARTERIES / ARTERIOLES		■		□		▨		■		□	
<u>PHARYNGEAL CARCINOMA</u>											
NO. SECTIONS: 2/2											
CAPILLARIES / VENULES		■		▨		▨		■		▨	
VEINS		■		▨		▨		■		▨	
ARTERIES / ARTERIOLES		■		□		▨		■		□	
<u>OSTEOSARCOMA</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■		▨ ^S		▨		□		□	
VEINS		■		□		▨		□		□	
ARTERIES / ARTERIOLES		⊗		⊗		⊗		⊗		⊗	
<u>ADENOCARCINOMA</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■		□ ^S		▨		▨ ^S		▨ ^S	
VEINS		■		□ ^S		▨		□		□	
ARTERIES / ARTERIOLES		⊗		⊗		⊗		⊗		⊗	
<u>MELANOMA</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■		▨		▨		▨ ^S		□	
VEINS		■		▨		▨		▨ ^S		□	
ARTERIES / ARTERIOLES		⊗		⊗		⊗		⊗		⊗	
<u>LUNG CARCINOMA</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■		▨		▨		▨ ^S		▨ ^S	
VEINS		■		▨		▨		▨ ^S		▨ ^S	
ARTERIES / ARTERIOLES		⊗		⊗		⊗		⊗		⊗	
<u>ANGIOSARCOMA</u>											
NO. SECTIONS: 1/1											
CAPILLARIES / VENULES		■	■	□	■	■	■	▨ ^S	■	▨ ^S	▨
VEINS		■	■	□	■	■	■	▨ ^S	■	▨ ^S	▨
ARTERIES / ARTERIOLES		□	□	□	□	□	□	□	□	□	□

SUBSTITUTE SHEET

FIG.14A

	FVIII		1G4		2G11		ICAM		ELAM	
	TA	NTA	TA	NTA	TA	NTA	TA	NTA	TA	NTA
<u>CAECUM CARCINOMA #</u>										
NO. SECTIONS: 1/1										
CAPILLARIES / VENULES	■		■		■		▨		▨	S
VEINS	■		■		■		▨		□	
ARTERIES / ARTERIOLES	■		▨		▨		▨		□	
<u>COLONIC CARCINOMA</u>										
NO. SECTIONS: 3/3										
CAPILLARIES / VENULES	■		▨		■		■		S	▨
VEINS	■		▨		■		■		S	▨
ARTERIES / ARTERIOLES	■		□		□		■		S	□
<u>COLON FAMILIAL</u>										
<u>ADENOMATOUS POLYPOSIS</u>										
NO. SECTIONS: 2/2										
CAPILLARIES / VENULES	■		■		■		▨		S	▨ LA
VEINS	■		■		■		▨		S	▨ LA
ARTERIES / ARTERIOLES	⊗		⊗		⊗		⊗		⊗	
<u>SIGMOID CARCINOMA</u> ∅										
NO. SECTIONS: 3/3										
CAPILLARIES / VENULES	■		▨		▨		▨		▨	S
VEINS	■		▨		▨		▨		▨	S
ARTERIES / ARTERIOLES	■		□		□		▨		□	
<u>RECTUM CARCINOMA</u>										
NO. SECTIONS: 2/2										
CAPILLARIES / VENULES	■		▨		■		■		□	
VEINS	■		▨		■		■		□	
ARTERIES / ARTERIOLES	⊗		⊗		⊗		⊗		⊗	
<u>RECTO-SIGMOID CARCINOMA</u> ∞										
NO. SECTIONS: 1/1										
CAPILLARIES / VENULES	■		■		■		■		▨	LA
VEINS	■		■		■		■		□	
ARTERIES / ARTERIOLES	■		▨		▨		▨		□	
<u>OVARIAN CARCINOMA</u>										
NO. SECTIONS: 1/1										
CAPILLARIES / VENULES	■		▨		▨		▨		□	
VEINS	■		▨		▨		▨		□	
ARTERIES / ARTERIOLES	■		▨		▨		▨		□	

FIG.14B SUBSTITUTE SHEET

	FVIII		1G4		2G11		ICAM		ELAM	
	TA	NTA	TA	NTA	TA	NTA	TA	NTA	TA	NTA
BENIGN BREAST										
NO. SECTIONS: 6/7										
CAPILLARIES / VENULES	■		□		□		□	S	□	S
VEINS	■	LU	□	LU	□		□	S	□	S
ARTERIES / ARTERIOLES	⊗		⊗		⊗		⊗		⊗	
HODGKIN'S DISEASE										
NO. SECTIONS: 11/11										
CAPILLARIES / VENULES	■		■	□	■	□	□	□	□	S
VEINS	■		■	□	■	□	□	□	□	S
ARTERIES / ARTERIOLES	■		□	⊗	□	⊗	□	⊗	□	
LYMPHOMA B-CELL NHL										
NO. SECTIONS: 2/2										
CAPILLARIES / VENULES	■		□		□		□		□	S
VEINS	■		□		□		□		□	S
ARTERIES / ARTERIOLES	■		□		□		□		□	S

KEYS:

■: +++ □: ++ □: + □: +/- □: -

S = SELETIVE

⊗ = NOT SEEN


















































































LA = LEUCOCYTE ASSOCIATED.

LU = LUMINAL.

TA = TUMOUR ASSOCIATED.

NTA = NON-TUMOUR ASSOCIATED.

FIG.14C

	<u>FVIII</u>	<u>1G4</u>	<u>2G11</u>	<u>ICAM</u>	<u>ELAM</u>
<u>PLACENTA</u>					
NO. SECTIONS: 1/1				M F	
CAPILLARIES / VENULES	■	M  F	M  F	■ ■	
VEINS	■	M  F	M  F	■ ■	
ARTERIES / ARTERIOLES	■	M  F	M  F	■ ■	
<u>STOMACH MUSCLE</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■				
VEINS	■				
ARTERIES / ARTERIOLES					
<u>STOMACH MUCOSA</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■				
VEINS	■	LU 			
ARTERIES / ARTERIOLES					
<u>LUNG</u>					
NO. SECTIONS: 3/3					
CAPILLARIES / VENULES	■				 S
VEINS	■				
ARTERIES / ARTERIOLES	■				
<u>PROSTATE</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■	LU  S	LU 	■	 S
VEINS	■	LU  S	S 		 LU
ARTERIES / ARTERIOLES	■				
<u>SPLEEN</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■			■	
VEINS	■				
ARTERIES / ARTERIOLES	■				
<u>MUSCLE IN LEG</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■				
VEINS	■				
ARTERIES / ARTERIOLES	■				

SUBSTITUTE SHEET

FIG.15A

	<u>FVII</u>	<u>1G4</u>	<u>2G11</u>	<u>ICAM</u>	<u>ELAM</u>
<u>BREAST</u>					
NO. SECTIONS: 3/3					
CAPILLARIES / VENULES	■	▨	▨	▨	▨ S
VEINS	■	▨	▨	▨	▨ S
ARTERIES / ARTERIOLES	■	□	□	▨	□
<u>COLON</u>					
NO. SECTIONS: 1/2					
CAPILLARIES / VENULES	■	▨	▨	▨	▨ S
VEINS	■	▨	▨	▨	▨ S
ARTERIES / ARTERIOLES	■	▨	▨	□	□
<u>RECTO-SIGMOID</u> ∞					
NO. SECTIONS: 1/1		(ONE 0/V ▨)			
CAPILLARIES / VENULES	■	□	▨	■	□
VEINS	■	□	▨	■	□
ARTERIES / ARTERIOLES	■	□	□	▨	□
<u>CAECUM #</u>					
NO. SECTIONS: 2/2					
CAPILLARIES / VENULES	■	□	□	▨	▨ S
VEINS	■	▨	▨	□	□
ARTERIES / ARTERIOLES	■	□	□	□	□
<u>THYMUS</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	▨	▨	▨	▨	□
VEINS	▨	▨	▨	□	▨
ARTERIES / ARTERIOLES	⊗	⊗	⊗	⊗	⊗
<u>KIDNEY CORTX</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■	▨	▨	▨	□
VEINS	■	□	□	□	□
ARTERIES / ARTERIOLES	■	□	▨	□	□
<u>LIVER</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■	□	□	□	□
VEINS	■	▨	▨	□	□
ARTERIES / ARTERIOLES	■	□	▨	▨	□

FIG.15B

SUBSTITUTE SHEET

	<u>FVIII</u>	<u>1G4</u>	<u>2G11</u>	<u>1CAM</u>	<u>ELAM</u>
<u>PANCREAS</u>					
NO. SECTIONS: 1/1					
CAPILLARIES / VENULES	■	□	▨	▩	□
VEINS	■	□	▨	▩	□ S
ARTERIES / ARTERIOLES	■	□	▨	▩	□
<u>INFLAMMED TISSUES</u>					
<u>REACTIVE HYPERPLASIA</u>					
NO. SECTIONS: 1/2					
CAPILLARIES / VENULES	■	□ S	▨	▩	□ S
VEINS	■	□ S	▨	▩	□ S
ARTERIES / ARTERIOLES	■	□	□	□	□
<u>CAT SCRATCH FEVER (REACTIVE)</u>					
NO. SECTIONS: 1/1				LA NLA	LA NLA
CAPILLARIES / VENULES	■	▨	▨	▩	▩
VEINS	■	▨	▨	▩	▩
ARTERIES / ARTERIOLES	■	□	□	▩	▩
<u>COLON: ULCERATIVE COLITIS</u>					
NO. SECTIONS: 1/2					
CAPILLARIES / VENULES	■	▨	▨	▩	□
VEINS	■	□	▨	▩	□ S
ARTERIES / ARTERIOLES	■	□	▨	▩	□
<u>SIGMOID: ULCERATIVE COLITIS</u>					
NO. SECTIONS: 1/1		LA NLA	LA NLA		
CAPILLARIES / VENULES	■	▩	▩	▩	□
VEINS	■	▨	▨	▩	□
ARTERIES / ARTERIOLES	■	▨	▨	▩	□
<u>TONSIL</u>					
NO. SECTIONS: 3/4					
CAPILLARIES / VENULES	■	▨	■	■	□ S
VEINS	■	▨	■	■	□ S
ARTERIES / ARTERIOLES	■	□	▨	▩	□

KEYS:

■: +++ ▩: ++ ▨: + ▨: +/- □: -

S = SELECTIVE

X = NOT SEEN

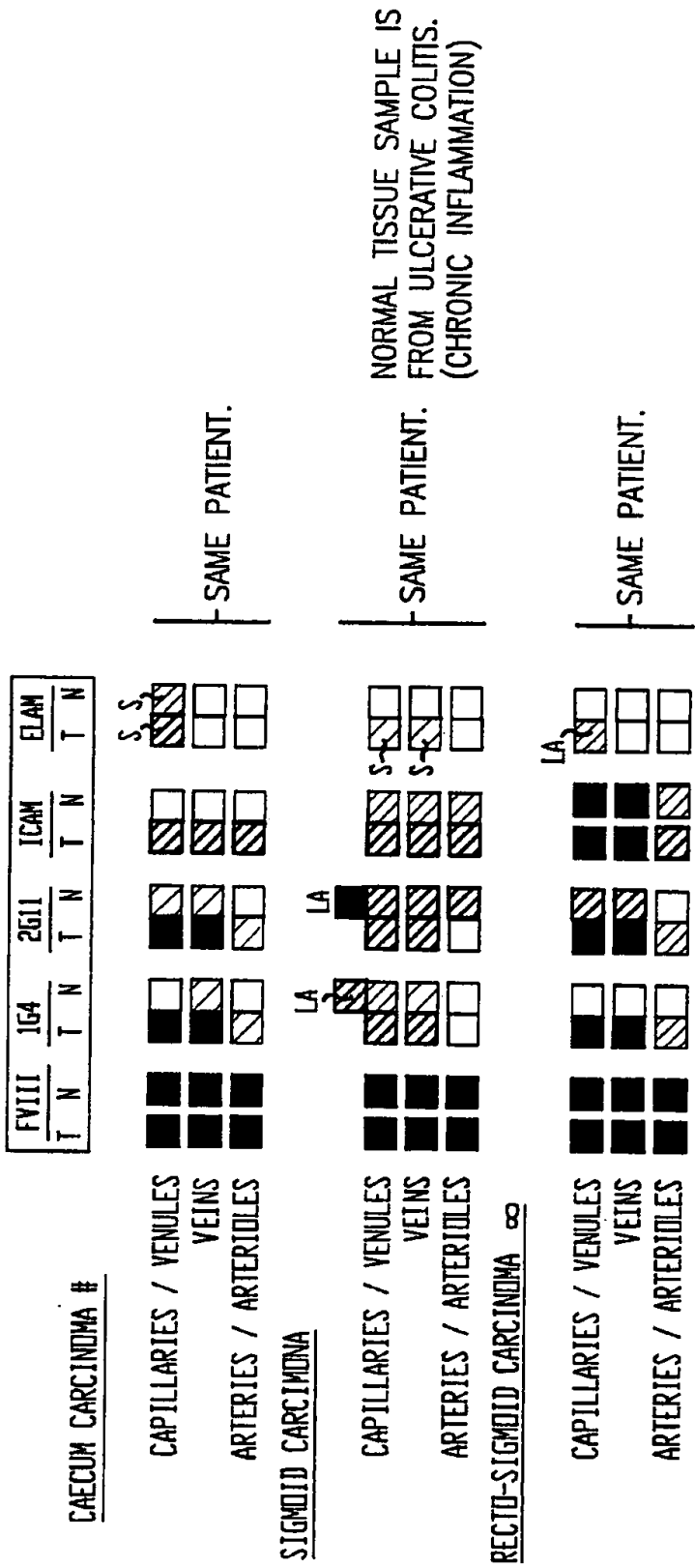
LA = LEUCOCYTE ASSOCIATED. F = FOETAL.

LU = LUMINAL.

NLA = NON-LEUCOCYTE ASSOCIATED

M = MATERNAL.

SUBSTITUTE SHEET**FIG.15C**



KEY.

■ = +++ ■ = ++ ■ = + ■ = +/- □ = - □ = NOT SEEN

S = SELECTED.

LA = LEUCOCYTE ASSOCIATED.

T = TUMOUR.

N = NORMAL.

FIG.16

INTERNATIONAL SEARCH REPORT

PCT/US 93/01956

International Application No.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K47/48; A61K49/00; A61K49/02

II. FIELDS SEARCHEDMinimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claims No. ¹³
X	INT. J. CANCER, VOL. 38, PAGE(S) 481-488, 1985, CH H.H. HAGEMEIET ET AL. 'A MONOCLONAL ANTIBODY REACTING WITH ENDOTHELIAL CELLS OF BUDDING VESSELS IN TUMORS AND INFLAMMATORY TISSUES, AND NON-REACTIVE WITH NORMAL ADULT TISSUES' cited in the application see abstract see page 487	1-56
Y	METH. ENZYMOLOGY, VOL. 93, PAGE(S) 280-333, 1983, GHOSE ET AL. 'Preparation of Antibody-linked Cytotoxic Agents.' cited in the application see page 325	1-56

¹⁰ Special categories of cited documents:^{"A"} document defining the general state of the art which is not considered to be of particular relevance^{"E"} earlier document but published on or after the international filing date^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)^{"O"} document referring to an oral disclosure, use, exhibition or other means^{"P"} document published prior to the international filing date but later than the priority date claimed^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.^{"A"} document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

03 JUNE 1993

Date of Mailing of this International Search Report

25.06.93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

DULLAART A.W.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
Y	<p>ANAL. BIOCHEM., VOL. 120, PAGE(S) 440-443, 1987, P.P. KNOWLES ET AL. 'Purification of immunotoxins containing Ricin A-Chain and Abrin A-Chain using Blue Sepharose CL-6B' cited in the application see the whole document</p> <p>---</p>	1-56
Y	<p>PROC. NATL. ACAD. SCI. U. S. A. , VOL. 84, PAGE(S) 9238-9242, 1987, US M.P. BEVILACQUA 'Identification of an inducible endothelial-leukocyte adhesion molecule.' cited in the application see abstract; table 1 see page 9241</p> <p>---</p>	1-56
X	<p>WO,A,9 013 300 (BIOGEN INC.) 15 November 1990 see abstract; claims 119-124</p> <p>---</p>	1-56
Y	<p>WO,A,9 012 585 (ONCOGEN LIMITED PARTNERSHIP) 1 November 1990 see examples</p> <p>---</p>	1-56
P,X	<p>WO,A,9 219 646 (THE ROCKEFELLER UNIVERSITY) 12 November 1992 see abstract see page 4, line 18 - page 5, line 14</p> <p>---</p>	1-56
P,Y	<p>WO,A,9 212 729 (CYTEL CORPORATION) 6 August 1992 see the whole document</p> <p>---</p>	1-56
P,Y	<p>INTERNATIONAL JOURNAL OF RADIATION APPLICATIONS AND INSTRUMENTATION PART B: NUCLEAR MEDICINE AND BIOLOGY vol. 19, no. 8, November 1992, OXFORD GB pages 897 - 902 T.S.T. WANG ET AL. 'Photoreactive 111In-cyclodextrin inclusion complex: a new heterobifunctional reagent for antibody labeling' see abstract</p> <p>-----</p>	1-56

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/01956

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
please see annex!
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
please see annex!
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9301956
SA 71138

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

03/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9013300	15-11-90	AU-A- 6049290	29-11-90
		CA-A- 2031518	29-10-90
		CA-A- 2063244	28-10-91
		EP-A- 0458911	04-12-91
		JP-T- 4502859	28-05-92
WO-A-9012585	01-11-90	US-A- 5202116	13-04-93
		CA-A- 2028825	11-10-90
		EP-A- 0422186	17-04-91
		JP-T- 4502769	21-05-92
WO-A-9219646	12-11-92	AU-A- 2025692	21-12-92
WO-A-9212729	06-08-92	AU-A- 1269092	27-08-92